THE UNIVERSITY *of* EDINBURGH

The role of transcription factor Pax6 in the development of the thalamocortical tract

By

James Clegg

Thesis submitted for the degree of doctor of philosophy at the University of Edinburgh

2013

Disclaimer

I (James Clegg) performed all of the experiments presented in this thesis unless otherwise clearly stated in the text. No part of this work has been or is being submitted for any other degree or qualification.

Signed:

Date:

Acknowledgements

First of all I would like to thank my supervisors Prof. David Price and Dr Martine Manuel. Their guidance, advise, instruction and patience over the last four years have made this thesis possible and I am very grateful for all the help they have given me.

I would like to thank the whole of the DBUG research group for creating a wonderful environment in which to work. In particular I would like to thank Chris Conway for instruction in tract tracing, Rowena Smith for advice and instruction on *in-situ* hybridisation, Viv Allison for assistance in histology and Michael Molinek for both his technical advice and constant supply of football banter. I would also like to thank Isabel Martin who, with Mike Molinek, created the chimeras used in chapter five. In addition I would like to say thank you to Trudi Gillespie for her help with confocal microscopy, and the Hugh Robson animal technicians without whom none of this work would have been possible.

Finally I would like to thank my family, particularly my Mum and Dad. Without their support over the years I would never have had the chance to progress this far in my academic career. I would also like to thank my fiancé Anneliese whose support and advice has been a great help over the course of my PhD.

Contents

Abbreviations

Amyg – Amygdaloid region

APCs – Apical progenitor cells

BPCs – Basal progenitor cells

CNS – Central nervous system

CTA – Corticothalamic axon

Ctx – Cortex

DiI - 1,1-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate

DiA - 4-(4-(dihexadecylamino)styryl)-N-methylpyridium iodide

dLGN – Dorsal lateral geniculate nucleus

DTB – Diencephalic-telencephalic boundary

ECM – Extra cellular matrix

GABA - Gamma-aminobutyric acid

GFP – Green fluorescent protein

GP – Globus pallidus

Hyp – Hypothalamus

ICZ – Internal capsule zone

LGE – Lateral ganglionic eminence

MGE – Medial ganglionic eminence

M1 – Primary motor cortex

Ncx – Neocortex

PCR – Polymerase chain reaction

***Pax6*^{cKO}** - *Pax6*$^{loxP/loxP}$ *Gsh2*^{Cre} conditional knockout embryo

***Pax6*$^{LacZ/KO}$**- *Pax6 LacZ* knockout embryo

***Pax6*$^{Sey/Sey}$** - Homozygous small eye (*Pax6* null) embryo

PSPB – Pallial-subpallial boundary

pTH-C – Caudal prethalamus

pTH-R – Rostral prethalamus

qRT-PCR – Quantitative real time polymerase chain reaction

RGCs – Radial glial cells

Shh – Sonic hedgehog

SVZ – Subventricular zone

TCA – Thalamocortical axon

Thal – Thalamus

TPOC – Tract of the postoptic commissure

VB – Ventrobasal complex

V Tel – Ventral telencephalon

VZ – Ventricular zone

V1 Primary visual cortex

WT – Wild type

ZLI – Zona limitans intrathalamica

Abstract

During development the nuclei of the thalamus form reciprocal connections with specific regions within the cortex. These connections give rise to the thalamocortical tract. The processes by which axons of the thalamocortical tract are guided to their target regions are poorly understood. It has been shown that diffusible or membrane bound factors can have a chemoattractive or chemorepulsive effect on the tip or growth cone of the axon. Thalamocortical axons may also be guided along 'pioneer' axon populations that form a scaffold along which axons may grow.

The transcription factor *Pax6* has been shown to have a role in a variety of developmental processes such as neuronal patterning, proliferation, migration and axon guidance. It is known that *Pax6* is involved in the development of the thalamocortical tract but its exact role is unknown. To explore the role that *Pax6* plays in the development of the thalamocortical tract I have used two different mouse models, the small eye ($Pax6^{Sey/Sey}$) mouse which lacks functional *Pax6*, and a conditional *Pax6* knock-out ($Pax6^{cKO}$) mouse made using a *Gsh2 Cre* line that specifically reduces *Pax6* expression in the ventral telencephalon and prethalamus.

Using the $Pax6^{Sey/Sey}$ mouse I show that thalamocortical axons do not enter the ventral telencephalon in the absence of Pax6 and that a small number of axons incorrectly enter the hypothalamus. In addition axons found within the ventral telencephalon of the mutant do not originate from the thalamus but instead originate from cells within the ventral telencephalon itself. I have found that the expression of guidance molecule *Robo2* is reduced in the $Pax6^{Sey/Sey}$ mouse, which may explain why thalamocortical axons enter the hypothalamus.

When *Pax6* expression is reduced at the prethalamus and ventral telencephalon using the $Pax6^{cKO}$ mouse I show that the majority of thalamocortical axons reach the cortex normally but some axons become disorganized within the thalamus. Pioneer axons which emanate from the prethalamus normally guide thalamocortical axons through the diencephalon but in the $Pax6^{cKO}$ I report that these axons are reduced which may explain the disorganization of thalamocortical axons within the thalamus.

Taken together the data from these two models demonstrate that for the thalamocortical tract to form normally *Pax6* expression is required in both the cells of the thalamus and in cells that lie along the route of the tract. In addition I provide evidence that *Pax6* may influence axon guidance by controlling the expression of guidance molecules and the development of pioneer axon tracts.

Chapter 1: General Introduction

1.1 Axon Guidance

1.1.1 Overview

The human brain is composed of billions of neurones. For the brain to function correctly these neurones must form synapses with other neurones that may be some distance away. The connections between neurones are achieved by axons, long thin projections from the cell body which may be covered by a sheath of myelin provided by astrocytes or Schwann cells. Bundles of myelinated axons form the large white matter tracts that can be seen on brain tissue sections or brain scans such as MRI. During development the axon extends from the cell body and grows towards its target cell. The route taken by the growing axon may be a complex one in three dimensions, for example a pyramidal cell located in one cerebral hemisphere may have to form a connection with a neurone in the opposite hemisphere. The route that this axon has to take is complex involving several turns; the axon also has to navigate across a large distance in cellular terms (several centimetres in the case of the human). The process by which the growing axon is directed to its target has been termed axon guidance.

The study of axon guidance will allow us to understand how neurones form connections with each other during development. As well as being of great interest in its own right this research may provide key insights into treatments for a range of medical conditions. Where axonal connections become damaged or broken by insults such as stroke or physical injury, knowledge of axon guidance may present methods by which these connections could be ‘re-wired’. Similarly an understanding of how the neuronal connections form may help develop better treatments for neurodegenerative diseases where these connections are progressively lost.

Currently we know very little about how the millions of different axons within the central nervous system are guided to their target cell. Over the last 25 years a number of genes have been identified which can directly influence the growth of the axon. It is currently unclear how an apparently small number of axon guidance genes can generate the seemingly infinite complexity of the axonal connections seen in the human brain. Even less is known about how the expression of these guidance

genes is controlled and coordinated by transcriptional regulators. Recent research has been directed at elucidating how the expression of axon guidance genes and cellular processes such as neuronal migration interact to guide axons of the major axonal tracts within the brain such as the thalamocortical tract and the corpus callosum.

1.1.2 The Growth Cone

As an axon extends through the brain it has been proposed that the axon detects changes in the extracellular environment and that these changes can dictate the direction in which axon growth will proceed. The structure that is particularly responsible for directing axon growth is the growth cone; this is a conical shaped structure found at the tip of the growing axon. Like so many other neuronal structures, the growth cone was first observed in the late nineteenth century by Spanish neuroanatomist and Nobel laureate Ramon y Cajal. He described the growth cone as 'protoplasm of conical form, endowed with amoeboid movements' (Cajal, 1890). The growth cone is a dynamic, motile structure which projects and retracts finger-like filopodia and sheet-like lammelipodia. The growth cone advances in a structured way. Firstly filopodia are extended from the body of the growth cone. If these projections do not retract or lift away from the substrate cytoplasm and organelles then move into the filopodia. The area behind the filopodia is then consolidated by the stabilisation of microtubules to form axonal shaft. This process is then continually repeated as the axon extends (Aletta and Greene, 1988; Sabry et al., 1991). The protrusion of filopodia and lamellipodia is largely dependent on the polymerisation of F-actin located throughout the periphery of the growth cone surrounding microtubules at the core of the growth cone (Fig. 1A) (Gallo and Letourneau, 2004). The preferential polymerisation of F-actin on one side of the growth cone in response to attractive signals from the environment and the stabilisation of the cytoskeletal network are thought to be the major mechanism by which growth cone turns towards these attractive signals. Conversely the disassembly of the F-actin network and the destabilisation of microtubules causes the retraction of the growth cone from areas of repulsion (Dent et al., 2011).

At the membrane of the growth cone are located guidance receptors which are responsible for detecting environmental cues to which the growth cone responds. These include members of the *Roundabout* (*Robo*) and *Unc* gene families. It is thought that these guidance receptors influence the growth cone by altering cytoskeletal dynamics via a second messengers such as cAMP (Song et al., 1997) and actin accessory proteins such as the Ableson tyrosine kinase (Bashaw et al., 2000).

The expression of particular guidance receptors are thought to confer upon the growth cone sensitivity to specific set of molecular guidance cues found along the route the growing axon must take. In this way axons can be ‘programmed’ to respond in a particular way to a set of environmental cues.

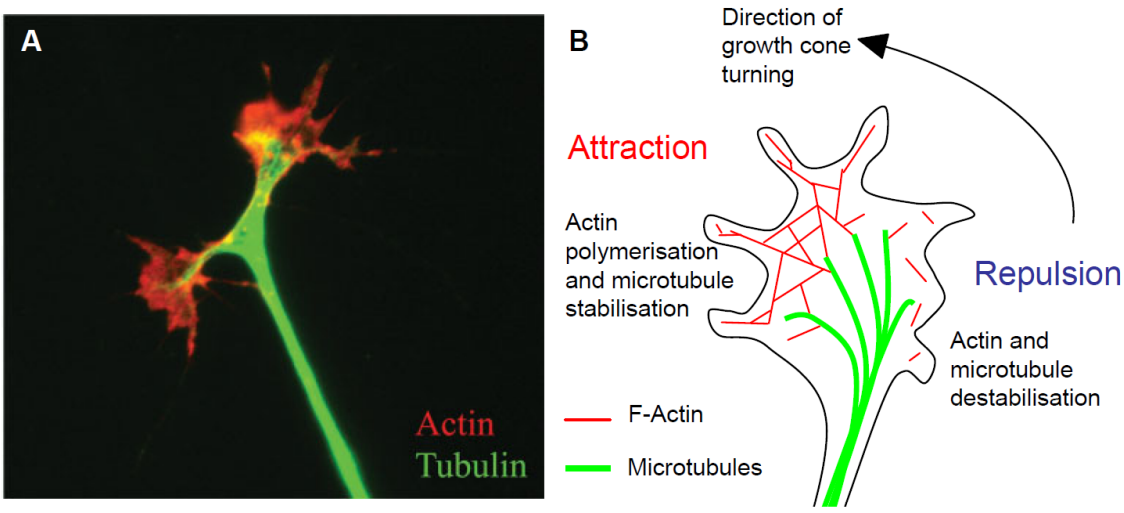


Figure 1. **The Growth Cone. (A)** Two growth cones of an embryonic chick dorsal root ganglion axon in culture. Immunohistochemistry reveals tubulin labelled microtubules in the axon shaft and the core of the growth cone (green), and actin at the periphery of the growth cone (red). From Gallo and Letourneau, 2004. **(B)** Schematic diagram illustrating the cytoskeletal processes involved in growth cone turning.

1.1.3 Contact Mediated Axon Guidance

Some of the earliest work on axon guidance revealed that the substrate upon which the axon grows can have an important role in axon guidance. Weiss showed that neurites of cultured neurons grew preferentially along scratches made in the culture dish, thus demonstrating that the surface upon which the axon grew could influence the direction of axon growth (Weiss, 1934). Later work on the grasshopper (*Lucusta migratoria*) showed that first two neurons that differentiate within the grasshopper limb bud designated as Ti1 extend their axons along a defined route through the limb toward the central nervous system (Bate, 1976). Axons from later born neurons follow the same path as the Ti1 pioneer axons to form a major nerve trunk within the adult limb using the pioneers as a substrate upon which to grow (Keshishian, 1980). The route taken by these pioneers is not a straight one, however: the axons make two near 90˚ turns as they traverse the limb bud. At specific points along its route the Ti1 axon makes contact with three cells designated F1, F2, and CT1 (Bentley and Keshishian, 1982). It was proposed that these cells act as intermediate targets for the Ti1 axon. These cells were termed "guidepost cells." Their importance was later demonstrated by a study which selectively destroyed the CT1 guidepost cells during limb bud development. It was found that in the absence of these cells the Ti1 axon does not make the second 90˚ turn toward the CNS and instead growth of the axon is either arrested at this point or continues abnormally within the limb bud. This demonstrated that contact between the growing axon and these guidepost cells were necessary for the correct guidance of pioneer axons in the grasshopper limb (Bentley and Caudy, 1983).

Further work identified cell adhesion molecules which play a role in this contact mediated axon guidance. *Fasciclin I* and *II* were shown to be expressed by a subsets of axons within the grasshopper and it was proposed that these genes provided a 'labelled pathway' upon which other axons could grow (Bastiani et al., 1986). Subsequent research showed that mutations in the *Fasciculin I* and *Ableson* genes resulted in major CNS axon tract abnormality, demonstrating the importance of *Fasciculin I* in guiding axons (Elkins et al., 1990).

There is evidence that contact mediated axon guidance involving both guidepost cells and pioneer axons are present in mammals as in invertebrates.

Pioneer axon populations are thought to play an important role in the guidance of several axon tracts within the mouse brain, one example is the corpus callosum where axons from the cingulate cortex are the first axons to cross the midline of the brain. These axons express guidance receptor *Neuropilin1* and are thought to pioneer the tract for later arriving axons originating from pyramidal cells within layer V of the cortical plate (Koester and O'Leary, 1994; Piper et al., 2009). Guidepost cells have been shown to be required for the correct development of the thalamocortical tract. Cells expressing guidance cue *Neuregulin1* migrate tangentially within the ventral telencephalon to provide a permissive 'corridor' along which thalamocortical axons can grow (Lopez-Bendito et al., 2006).

1.1.4 Molecular Guidance Cues

Following the observation by Cajal that growth cones were dynamic structures displaying ameboid-like movements he postulated that growth cones may be guided by gradients of chemical substances within the environment. This theory stated that as the growth cone grew through the environment it encountered concentration gradients of chemical substances and that the growth cone responded by turning towards or turning away from the source of these substances. The theory was termed 'chemotropism' but evidence for this form of axon guidance would not surface for almost a century.

Co-culture experiments performed in the late 1980s showed that the floor plate of the rat spinal cord had an attractive influence on commissural spinal cord axons despite the fact that the floor plate explants were not in contact with the axon growth cones. This proves that the floor plate must produce a diffusible substance which is detected by the growing axons (Placzek et al., 1990; Tessier-Lavigne et al., 1988). This substance was later characterised as *Netrin1* which was shown to be expressed along the length of the floor plate (Serafini et al., 1994).

In *Netrin1* deficient mice the attractive effect of the floor plate in culture was abolished and the commissural axons which normally cross through the floor plate *en route* to the opposite half of the spinal cord no longer do so (Serafini et al., 1996). This data suggested that *Netrin1* expression was primarily responsible for attracting

commissural axons to the floor plate. This though poses the question why do commissural axons leave the floor plate once they get there? An answer to this question arose from work on *Drosophila melanogaster*. The extracellular protein *Slit,* was identified in *Drosophila* and shown to be expressed at the midline during development (Rothberg et al., 1988). Drosophila where *Slit* expression is reduced display midline commissural axon defects in a similar way to *Netrin1* mutants suggesting a role for *Slit* in regulating the development of commissural axon tracts (Rothberg et al., 1990). The *Roundabout* (*Robo*) receptor was shown to be the receptor for extracellular *Slit* protein and *Robo* mutant *Drosphila* also show midline commissural axon defects. The spatial regulation of *Robo* expression was shown to be a key factor in determining the ability of axons to cross the midline (Kidd et al., 1999; Seeger et al., 1993). *Slit* was shown to be expressed in the floor plate of the rat spinal cord in a similar pattern to *Netrin1*, and culture experiments demonstrated that Slit protein could act as a repellent to spinal cord axons (Brose et al., 1999). It was proposed that *Slit*-mediated repulsion was the mechanism by which axons were repelled from the floor plate once commissural axons had crossed the midline. This was achieved by the upregulation of expression of the S*lit* receptor *Robo* once the midline had been reached coupled with a simultaneous reduction in expression of *Netrin1* receptor *DCC* (Shirasaki et al., 1998; Stein and Tessier-Lavigne, 2001). So the crossing of the midline by spinal cord axons was achieved by the temporal regulation of growth cone responsiveness to attractive and repulsive cues present at the floor plate (Fig. 2). This interplay between the expression of diffusible guidance cues and the ability of the growth cone to respond to these cues is important for the development of other commissural axon pathways such as the optic tract and the cerebellofugal tract (Plump et al., 2002; Tamada et al., 2008).

Changes in the expression of guidance receptors at the growth cone can allow the growing axon to respond to different guidance cues at different times. But the expression of different guidance receptors can also elicit different growth cone responses to the same guidance cue. Examples of this are two receptors for *Netrin1*; *DCC* and the *Unc5* family of proteins. *Netrin/DCC* signalling is known to cause an attractive response in growing axons (Keino-Masu et al., 1996) but this attractive response can be converted to repulsion if *Unc5* is also expressed at the growth cone

(Hong et al., 1999). This bi-functionality of *Netrin1* allows for the generation of greater complexity and is one way in which a fairly small number of axon guidance cues may guide the vast number of different axons within the CNS.

In addition to the *Netrins*, the *Slits* and their receptors a number of other families of guidance cues have been identified (summarised, Fig. 3). These include the Semaphorins some of which act as diffusible and membrane bound guidance cues (Kolodkin et al., 1993). Semaphorin signalling occurs through their cell surface receptors Plexin and Neuropilin (Chen et al., 1997; Winberg et al., 1998). Semaphorin/Plexin/Neuropilin signalling is involved in the development of several axon tracts within the mammalian brain including the corpus callosum and the thalamocortical tract (Leighton et al., 2001; Piper et al., 2009). *Ephrins* and *Ephs* are membrane bound guidance cues thought to be particularly important for maintaining the correct topographical arrangement of axons as they extend to their target. This is the case for the thalamocortical tract where expression gradients of *Ephrins* both within the thalamus and cortex are proposed to direct axons from particular thalamic nuclei to specific areas of the cortex (Dufour et al., 2003). Several other guidance cues have been characterised, these include *Draxin, Netrin1* receptor DSCAM and protocadherin *Celsr3* (Ahmed et al., 2011; Islam et al., 2009; Tissir et al., 2005). Morphogens such as Wnts, FGFs, BMPs and Sonic Hedgehog which are heavily involved in forebrain development and regionalisation can act as guidance cues (Sanchez-Camacho and Bovolenta, 2009).

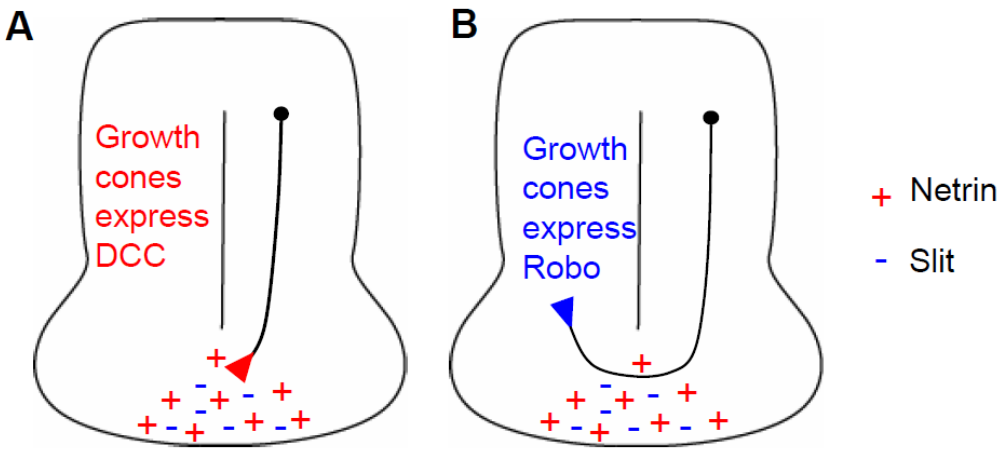


Figure 2. Attraction and repulsion at the floor plate of the spinal cord. (A, B) Schematic representations of a section through the rodent spinal cord. **(A)** Attractive guidance cue *Netrin* and repulsive cue *Slit* are expressed at the floor plate. Initially commissural axons express *Netrin* receptor *DCC* are attracted to the floor plate. **(B)** After crossing the midline axons express *Slit* receptor *Robo* and are repelled from the floor plate.

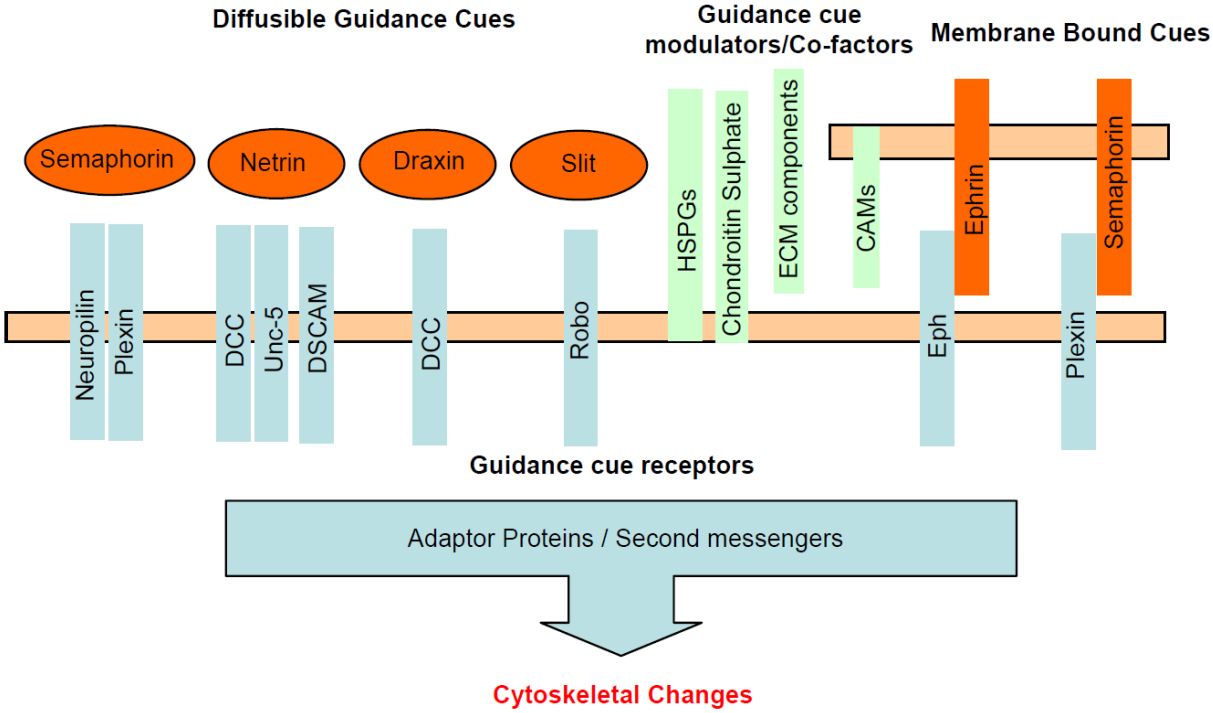


Figure 3. Summary of the major axon guidance cues and their receptors.

1.1.5 Axon guidance and the extracellular matrix

Elements of the extracellular matrix (ECM) can also play a major role in guiding axons. The ECM is made up of a number of proteins and proteoglycans that are secreted by cells into the interstitial space. These include collagen, laminin and heparan sulphate proteoglycans. The ECM fulfils a number of roles including physical support for cells and tissues, regulation of morphogen diffusion and tissue repair as well as providing a substrate for cell and axon growth. Cells are anchored to the ECM via *integrin* receptors located at the plasma membrane. Several studies have demonstrated that axon guidance cues including *Netrin* and *Semaphorins* can affect the activity of *integrin* receptors (Nakamoto et al., 2004), this may influence the binding of the growth cone to the ECM and therefore traction of the growth cone through the brain. Constituents of the ECM can also modulate the effect of guidance cues. *Netrin1* has an attractive effect on Xenopus retinal ganglion cell axons cultured upon a *Fibronectin* substrate but this attraction is converted to repulsion when Laminin is the substrate (Hopker et al., 1999). Other ECM components may be involved in the binding of guidance cues to their receptors acting as co-receptors. Heparan sulphate proteoglycans are thought to be required for binding of diffusible axon guidance cue *Slit* to its receptor on the growth cone *Robo* (Hussain et al., 2006) and may be involved in *Netrin1* and *Semaphorin* signalling (Kastenhuber et al., 2009). Another group of proteoglycans, Chondroitin Sulphates, may also be involved the signalling of certain *Semaphorins* (Kantor et al., 2004).

1.2 The development of the murine thalamocortical tract

1.2.1 Overview

The thalamus is a major relay centre in the brain. Sensory information from the periphery first arrives in the brain at the thalamus, and is then relayed to the relevant area of the cortex. Sensory input to the thalamus includes visual, auditory and somatosensory information. In order for this sensory information to be transmitted from the thalamus to the cortex, a large axonal tract forms during development to connect the two structures; this is the thalamocortical tract. The route taken by the growing thalamocortical axons (TCAs) is a complex one in three dimensions, and involves navigating through a number of different regions within the developing brain. As the TCAs extend through the brain they are confronted with a number of 'choice points' where the axons must choose to grow in one direction or another. By examining these choice points we can attempt to elucidate the molecular processes involved in guidance decisions. This makes the thalamocortical tract an excellent model by which to study axon guidance.

1.2.2 The development of the thalamus

After the neural plate folds to form the neural tube, the rostral end of the tube swells to form the brain, while the caudal region will become the spinal cord. The early brain is divided into three vesicles: the prosencephalic vesicle which will form the forebrain, the mesencephalic vesicle which will form the midbrain and the rhombencephalic vesicle which will form the hindbrain. As development proceeds the prosencephalic vesicle is further divided into a diencephalic vesicle, flanked by two telencephalic vesicles. The telencephalic vesicles will give rise to the structures of the telencephalon such as the cortex and the basal ganglia while the diencephalic vesicle will form the structures of the diencephalon such as the thalamus and hypothalamus. According to the prosomeric model of development, the developing diencephalon can be subdivided along the longitudinal axis of the neural tube into three prosomeres, designated P1, P2 and P3. P1 will become the pretectum, P2 will give rise to the thalamus and P3 will form the prethalamus (Puelles and Rubenstein,

2003). Between P2 and P3 lies the zona limitans intrathalamica (ZLI) which expresses morphogens such as *Sonic hedgehog* (*Shh*) and members of the *FGF* and *Wnt* gene families. Loss of *Shh* expression at the ZLI causes molecular patterning abnormalities and cell fate changes within the thalamus and prethalamus demonstrating the important role *Shh* has in the development of these two structures (Vue et al., 2009).

From around embryonic day 9 (E9) in mouse development gene expression studies have identified that the thalamus becomes divided into a number of molecularly distinct regions. Initially the progenitor cells that will give rise to the thalamus are divided into two regions; the large pTH-C which expresses transcription factor *Ngn2* and the smaller pTH-R which expresses *Nkx2.2* and *Mash1* (Vue et al., 2007). At E12.5 the first TCAs extend from postmitotic neurons within the thalamus; by this stage the thalamus has become further divided into several regions which express different combinations of marker genes such as *Gbx2* and *Lhx2*. Later in development these marker genes can be used to identify the nuclei of the thalamus, which become morphologically distinct by postnatal day 2 (Nakagawa and O'Leary, 2001). Each thalamic nucleus forms connections with a specific region of the cortex and these connections are made at the same time as the nuclei are developing, meaning it is highly likely that the two processes are linked.

1.2.3 The formation of the thalamocortical tract

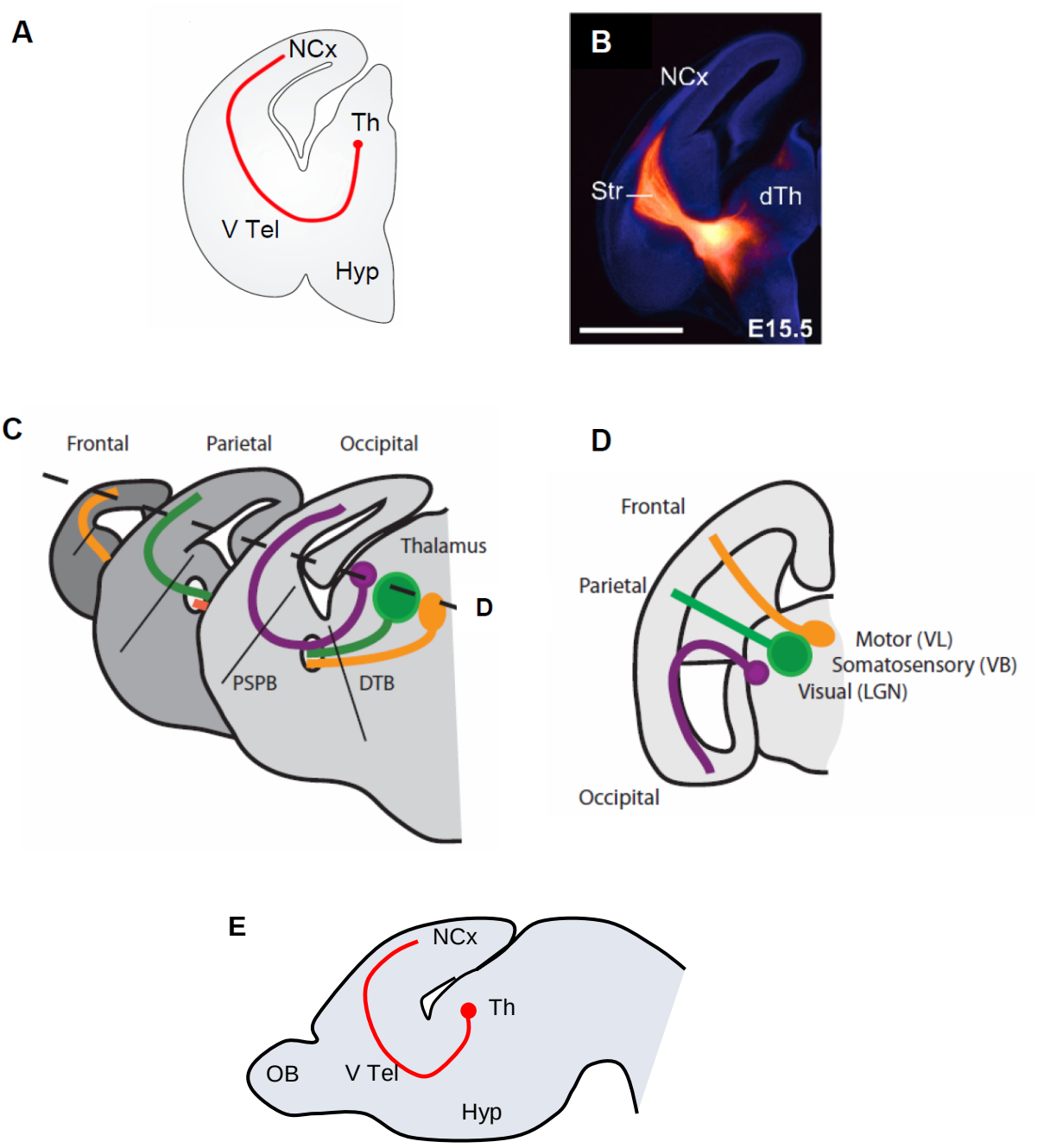
The thalamocortical tract in the mouse begins to form at around E12.5. At this stage the first TCAs extend from postmitotic neurons found in the lateral portion of the thalamus. The TCAs extend in a ventral direction, crossing the ZLI to reach the prethalamus. From here the TCAs head further ventrally but turn laterally before reaching the hypothalamus. As they turn the TCAs cross the diencephalic-telencephalic boundary (DTB) and reach the ventral telencephalon by E13.5. The TCAs extend in a lateral direction through the internal capsule zone (ICZ) before turning once again, this time in a dorsal direction towards the developing cortex. To reach the cortex the TCAs cross the pallial-subpallial boundary (PSPB), an important morphological and gene expression boundary separating the dorsal palium (which

will form the cortex) from the ventral subpalium (which will form the basal ganglia). TCAs begin to cross this boundary at E14.5 and extend through the intermediate zone of the cortex. The axons will reach the correct region of the cortex by about E18.5. During postnatal development branches of TCAs extend into the cortical plate and by postnatal day 2 they reach layer IV where they will form synapses with cortical neurons (Fig 4).

The thalamocortical tract does not form in a flat two dimensional plane as may be suggested by the above description, but is a more complex three dimensional structure. As TCAs leave the thalamus and enter the telencephalon they are channelled through a narrow 'corridor', but then fan out in both rostral and caudal directions. Upon reaching the cortex the TCAs are widely distributed along the rostro-caudal axis to different cortical regions. The distribution of the TCAs as they extend to the cortex is not random, it is highly ordered. As TCAs project from the various nuclei of the thalamus the topographical arrangement of these axons is maintained along the length of the tract. The cortical area to which TCAs project reflects the region of the thalamus from which they originated, broadly speaking medial thalamic regions project to rostral/frontal cortical areas while lateral thalamic regions project to caudal/parietal areas (Fig 4C, D) (Lopez-Bendito and Molnar, 2003; Price et al., 2012; Price et al., 2006).

At the same time as the thalamocortical tract is developing the cortex forms reciprocal connections with the thalamus; this is the corticothalamic or corticofugal tract. The first corticothalamic axons (CTAs) extend from projection neurones within the cortical plate at around E12, and cross the PSPB to reach the ventral telencephalon by E14.5. Within the ICZ, CTAs come into contact with TCAs, from this point onward the CTAs closely follow the route of the thalamocortical tract back to its source, reaching the thalamus by E17.5 (Auladell et al., 2000).

Fig. 4. The thalamocortical tract. (A) Schematic representation of the route of the thalamocortical tract (red line) through the embryonic mouse brain, as seen in a coronal section. **(B)** Coronal section through a mouse brain at E15.5 showing the thalamocortical tract labelled with a carbocyanine dye (taken from Little et al, 2009). **(C)** Schematic diagram illustrating the topographic arrangement of TCAs that project from the various nuclei of the thalamus to specific regions of the cortex. **(D)** Horizontal section at the plane of the dashed line in C indicating the arrangement of thalamocortical projections (taken from Price et al, 2012). **(E)** Schematic representation of the route of the thalamocortical tract (red line) as seen in a parasagittal section. Abbreviations: Hyp, hypothalamus; NCx, neocortex; OB, olfactory bulb; Str, striatum; Th, Thalamus; Vtel, ventral telencephalon.


A
NCx
Th
V Tel
Hyp
B
NCx
Str
dTh
E15.5
C
Frontal
Parietal
Occipital
Thalamus
D
PSPB
DTB
D
Frontal
Parietal
Motor (VL)
Somatosensory (VB)
Visual (LGN)
Occipital
E
NCx
Th
OB
V Tel
Hyp

1.2.4 Guidance of thalamocortical axons

Although it is still unclear exactly how TCAs navigate from the thalamus to the cortex, we do know of a number of mechanisms by which these axons are guided. Firstly a number of diffusible and membrane bound molecular guidance cues are involved in TCA guidance. Knockout studies analysing a number of different mice deficient for certain guidance genes have shown that several guidance cues such as *Netrin1*, *Slits* and *Semaphorins* are involved in the development of the thalamocortical tract (Bagri et al., 2002; Braisted et al., 2000; Leighton et al., 2001). Secondly, certain populations of cells found along the route of the thalamocortical tract extend 'pioneer axons' which are proposed to provide support and guidance for navigating TCAs (Mitrofanis and Guillery, 1993). Thirdly cells within the ventral telencephalon are thought to act as 'guidepost cells' providing permissive territory through which TCAs are able to grow. The migration of these cells to the correct position during development is crucial to the correct formation of the thalamocortical tract (Lopez-Bendito et al., 2006). Over the rest of this section we will examine these guidance mechanisms in more detail.

1.2.5 Guidance Cues

Netrin1

Diffusible molecular guidance cues can act in an attractive or repulsive fashion, repelling the growth cone from a territory the axon should not enter or attracting the axon towards a region it should enter. *Netrin1* was the first identified diffusible guidance cue; it was shown to be a floor plate attractant for commissural spinal cord axons (Placzek et al., 1990; Tessier-Lavigne et al., 1988). *Netrin1* has also been implicated in the development of the thalamocortical tract. *Netrin1* has been show to act as an attractant to TCAs in culture and it is expressed by cells of the internal capsule zone within the ventral telencephalon, while *Netrin* receptors DCC, *Unc5c* and *Neogenin* are expressed by cells within the thalamus. In *Netrin1* knockout mice the thalamocortical tract does not develop correctly; TCAs appear disorganised within the internal capsule zone and the number of TCAs projecting to the cortex is reduced (Braisted et al., 2000; Serafini et al., 1996; Shu et al., 2000). These findings

suggested that *Netrin1* was the attractant primarily responsible for causing TCAs to turn towards the telencephalon *en route* to the cortex; later research has shown, however, that *Netrin1* plays a more nuanced role in TCA guidance. Further culture studies revealed that while *Netrin1* was indeed an attractant for TCAs originating from the posterior half of the thalamus, it acted as a repellent for TCAs from the anterior half. This difference in response to *Netrin1* is likely due to the fact that in posterior regions of the thalamus *Netrin* receptor DCC is expressed alone while in the anterior half both DCC and Unc5c are expressed. DCC alone is known to mediate the attractive response to *Netrin1* whereas DCC/Unc5c complexes mediate repulsion (Hong et al., 1999). Furthermore it was shown that *Netrin1* expression was not uniform throughout the internal capsule zone but was in fact expressed as a rostral^{High} to caudal^{Low} gradient (Bonnin et al., 2007; Powell et al., 2008). Taken together these data suggest that *Netrin1* is primarily involved in maintaining the topographic arrangement of TCAs rather than acting as a simple attractant to all thalamic axons. Further evidence for this hypothesis came from a series of elegant culture experiments whereby the ICZ of cultured thalamocortical slice preparations was flipped in a rostocaudal direction. This flipping (and therefore reversal in the direction of the *Netrin1* expression gradient) caused posterior thalamus TCAs, which normally extend through the caudal part of the ICZ, to instead grow more rostrally (Bielle et al., 2011b). This is consistent with a role for *Netrin1* as a regulator of the topography of TCAs as they project through the ventral telencephalon.

Slit/Robo

The repulsive guidance cue *Slit* and its receptor *Roundabout* (*Robo*) were first identified in *Drosophila*. *Slit* was shown to act as a midline repellent for commissural axons both in *Drosophila* and rodents (Holmes et al., 1998; Kidd et al., 1999; Rothberg et al., 1990). Culture experiments have identified that *Slit2* has a repulsive effect on spinal cord axons and various different forebrain axon populations such as olfactory bulb axons and callosal axons (Brose et al., 1999; Nguyen Ba-Charvet et al., 1999; Shu et al., 2003).

Slit1 is expressed at the midline of the forebrain and within the hypothalamus. It is also found at the ventricular zone of the ventral telencephalon. *Slit2* is expressed at the midline and is strongly expressed at the hypothalamus. *Robo1* and *Robo2* are

both expressed within the thalamus. The hypothalamus is known to be a region that is repulsive to TCAs (Braisted et al., 1999). The pattern of expression of both the *Slit* ligand and its *Robo* receptor suggests that *Slit/Robo* signalling may be responsible for this repulsion, causing TCAs to turn away from the hypothalamus and towards the telencephalon. Culture experiments have shown that *Slit2* indeed has a repulsive effect on TCAs and that unlike *Netrin1* this effect is the same for TCAs originating from both the posterior and anterior halves of the thalamus (Bonnin et al., 2007). In mice deficient in *Slit2* TCAs do not navigate normally; instead of turning laterally towards the telencephalon a subset of axons invade the hypothalamus. In mice where both *Slit1* and *Slit2* expression is lost this effect is magnified with a larger number of TCAs misrouted to the hypothalamus (Bagri et al., 2002). Another study has shown that when *Robo1* and *Robo2* are knocked out TCAs also enter the hypothalamus in a similar fashion to that seen in $Slit1^{-/-}Slit2^{-/-}$ mice (Lopez-Bendito et al., 2007). Further co-culture studies revealed that the repulsive effect of the hypothalamus on TCAs could be abolished by the addition of a *Slit* function blocking agent in the culture medium indicating that *Slit/Robo* signalling is responsible for the repulsive character of the hypothalamus (Braisted et al., 2009) . These experiments using both culture techniques and transgenic approaches confirm that the expression of *Slit* and *Robo* genes co-operate to cause repulsion of TCAs away from the hypothalamus and towards the telencephalon.

Semaphorins

Semaphorins are known to act both as cell membrane bound and diffusible axon guidance cues (Kolodkin et al., 1993). *Sema6A* is expressed within the thalamus during thalamocortical tract development. *Sema6A* mutant mice display several TCA guidance defects. Firstly TCAs which originate from the dorsal lateral geniculate nucleus (dLGN) are misrouted within the ventral telencephalon forming an axon bundle that projects ventrally rather than entering the internal capsule. Secondly the topography of the thalamocortical projections to the cortex is altered, with TCAs from the ventrobasal complex (VB) projecting into areas normally innervated by TCAs from the dLGN (Leighton et al., 2001; Little et al., 2009). This demonstrates that for TCAs to be guided correctly, *Sema6A* expression is required in thalamic projection neurons, particularly those of the dLGN.

Sema3A is known to act as a repulsive guidance cue for both TCAs and CTAs (Bagnard et al., 2001). It is expressed within the ventral telencephalon in a caudal^{High} to rostral^{Low} gradient in a similar manner to *Netrin1* (Skaliora et al., 1998). The receptor for *Sema3A* at the growth cone is *Neuropilin1*. In order for *Sema3A* to cause growth cone turning, there is a requirement for cell adhesion molecule close homologue of L1 (CHL1) which forms a complex with the *Neuropilin* receptor. In mice lacking CHL1 the topography of thalamocortical projections to the cortex is disrupted (Wright et al., 2007). This demonstrates the role that *Sema3A* plays in maintaining the topographic arrangement of TCAs as they project through the ventral telencephalon. It also further highlights the importance of the ventral telencephalon in this process.

Eph/Ephrins

It has already been mentioned that several different guidance cues are involved in regulating the topography of thalamic projections to the cortex. Another family of genes involved in this process are the *Ephrins*, which are membrane bound ligands for the *Eph* receptor tyrosine kinase family. It has been shown that *Ephrins* can guide cultured retinal axons (Drescher et al., 1995) and that expression of *Ephrins* and their *Eph* receptors can control the topographic arrangement of retinal and hippocampal projections (Cheng et al., 1995; Gao et al., 1996). *EphrinA5* is expressed in a gradient in both the cortex and the ventral telencephalon, while the receptors *EphA3*, *EphA4*, and *EphA7* are expressed in gradients across the thalamus. Analysis of mutant mice deficient for various combinations of these *Eph* and *Ephrin* genes show that the graded expression of receptor and ligand are required for the correct topographical arrangement of TCAs as they project to the cortex. The *EphrinA5* gradient present in the ventral telencephalon in combination with *Eph* receptor gradients across the thalamus determine the rostro-caudal position of TCAs as they navigate through this region. The *EphrinA5* expression gradient in the cortex combined with the *EphA4* gradient in the thalamus control the precise inter-areal topography of TCAs within the cortex itself (Dufour et al., 2006; Dufour et al., 2003).

1.2.6 Pioneer Axons.

Early research into axon guidance using the developing grasshopper limb showed that after the first axons extended from neurons within the limb bud towards the CNS, axons from later born neurons would follow the same route (Keshishian, 1980). These 'pioneer axons' acted to provide a scaffold that other axons could use to reach their target. In mammals the first identified axons to perform this role were axons from cells of the preplate within the developing cortex, which extend axons into the ventral telencephalon and act as pioneers for later descending cortical axons (McConnell et al., 1989). Along the route of the thalamocortical tract there are several largely transient populations of neurons that extend pioneer axons in order to aid the guidance of growing TCAs to the cortex. Within the diencephalon there are a population of cells located at the prethalamic reticular nucleus that extend axons dorsally into the thalamus at around E12 in the mouse, shortly before the first TCAs grow out of the thalamus (Braisted et al., 1999; Mitrofanis and Baker, 1993). It is thought that these axons pioneer the initial segment of the thalamocortical tract, providing support and guidance to TCAs as they cross the ZLI to reach the prethalamus. Another group of pioneer axons extend to the thalamus from a population of cells found at the ICZ of the ventral telencephalon. These axons are present from E12.5 and are in a position to guide axons from the thalamus as far as the internal capsule (Metin and Godement, 1996; Molnar and Cordery, 1999). These pioneer axon tracts are also present in close proximity to the thalamocortical tract in both reptiles and marsupials, suggesting that these axons play an evolutionarily conserved role in thalamocortical tract development (Cordery and Molnar, 1999; Molnar et al., 1998b).

Observations from mutant mice lacking certain transcription factors have provided evidence that these pioneer axon tracts are required for the normal development of the thalamocortical tract. In mice lacking the transcription factor *Mash1* the thalamocortical tract is malformed. TCAs do not cross the DTB to reach the telencephalon but remain within the diencephalon. Tract tracing experiments have shown that the pioneer axon tract which originates from the prethalamus does form in *Mash1*$^{-/-}$ mice but the tract emanating from the ICZ does not (Tuttle et al., 1999). This suggests that the ICZ pioneer axons may play a particularly important

role in allowing TCAs to cross the DTB. *Emx2*$^{-/-}$ mice also display thalamocortical pathfinding errors. Although TCAs reach the cortex in these mice, they are deflected ventrally as they navigate through the ventral telencephalon. Interestingly the cells which normally extend pioneer axons from the ICZ are displayed ventrally, to the same region through which TCAs will aberrantly grow. This suggests that the pioneer axons possess the ability to guide TCAs even when the route of the pioneers is altered (Lopez-Bendito et al., 2002).

In addition to the pioneer axon populations it has also been postulated that CTAs and TCAs may be able to guide each other after they meet within the ventral telencephalon. This has been termed the 'handshake hypothesis' (Blakemore and Molnar, 1990; Molnar and Blakemore, 1995). After reaching the ventral telencephalon TCAs navigate to the cortex following the route of the corticothalamic tract almost exactly. Individual TCAs also grow in very close proximity to CTAs suggesting that the two groups of axons could be providing guidance for each other (Molnar et al., 1998a). Further evidence for a role for CTAs in the guidance of TCAs comes from a study using conditional mutagenesis to knockout tumour suppressor gene *adenomatous polyposis coli* (APC) specifically within the cortex. APC is required for axonal outgrowth, therefore when it is removed from the cortex CTAs fail to form. In the absence of CTAs, TCAs are unable to cross the PSPB and reach the cortex. Culture experiments showed that replacing the APC mutant cortex with control cortex (and therefore CTAs) allowed TCAs to once again reach the cortex (Chen et al., 2012). These findings support the hypothesis that CTAs are required for the correct guidance of TCAs from the ventral telencephalon to the cortex, and that CTAs may be of particular importance for the crossing of the PSPB.

1.2.7 Guidepost cells and the 'corridor'

Research into the guidance of pioneer axons within the grasshopper limb bud has shown that so called 'guidepost cells' which the axons contacts along its route provide important guidance information, and that when these cells are lost guidance is disrupted (Bentley and Caudy, 1983). Recent studies have identified a population

of cells within the ventral telencephalon which act as guidepost cells for navigating TCAs.

During development cells from the LGE migrate tangentially into the MGE, forming a 'corridor' of cells within the MGE which express marker genes of LGE cells such as *Ebf1* and *Islet1* (Wichterle et al., 2003). These corridor cells also express the cell membrane bound guidance cue *Neuregulin1* which promotes axon growth and cellular migration (Flames et al., 2004) . The MGE and globus pallidus (GP) are non-permissive to TCAs, and this *Neuregulin1* expressing corridor provides permissive territory through which TCAs can grow. This tangential migration occurs at around E12.5, shortly before TCAs cross the DTB. *Ex vivo* culture experiments have shown that when the migration of cells from the LGE is blocked, preventing corridor formation, TCAs cannot advance through the MGE. This demonstrates that corridor formation is required for TCAs to navigate through the MGE (Lopez-Bendito et al., 2006). It appears that the migration of the corridor cells acts to control the timing of TCA growth through the ventral telencephalon and channels the axons into a tight bundle in order to direct growth towards the cortex.

A recent study has shown that corridor-like cells can be observed in mammalian, avian and reptilian species; however, corridor cells are only able to guide TCAs in mammals. This is because the corridor cells of avian species do not migrate to a position where they can contact TCAs entering the telencephalon. In avian species like the chicken TCAs cross the ventral telencephalon ventral to the GP following a so called 'external' route to the cortex in contrast to mammals where TCAs follow an 'internal' route above the GP. There is evidence that the 'opening' of this internal route in mammals requires *Slit/Robo* signalling which controls the precise positioning of corridor cells as they migrate from the LGE (Bielle et al., 2011a).

1.3 The role of Pax6 in the development of the forebrain.

1.3.1 Overview

The *Pax6* gene encodes a transcription factor, which is a member of the paired-box (*pax*) gene family. *Pax6* contains two DNA binding domains, a paired domain and a homeobox domain. *Pax6* is highly evolutionarily conserved; the coding region of the *Pax6* gene is 100% identical in humans and rodents, while the *Drosphila melanogaster* homologue of *Pax6*, *eyeless*, is 95% identical (Quiring et al., 1994). In addition to the sequence conservation, *Pax6* function is also conserved in across vertebrate and invertebrate species.

In *Drosophila*, loss of the *Pax6* homologue *eyeless* causes a complete failure in eye development. In the mouse, *Pax6* was first identified as the gene responsible for causing the small eye (*sey*) craniofacial phenotype confirming its conserved role in eye development (Hill et al., 1991; Hogan et al., 1988; Ton et al., 1991). The human condition aniridia causes developmental eye defects due to a heterozygous loss of *Pax6* function, similar to that seen in the small eye mouse. Aniridia in humans is characterised by a loss of the iris along with a range of other eye conditions such as nystagmus and cataracts (Glaser et al., 1994).

In mice *Pax6* has been show to be expressed within the eye and specific regions of the CNS during development, which is consistent with a role in eye and brain development (Walther and Gruss, 1991). In addition to the failure of eye formation, the homozygous loss of *Pax6* (*Pax6*$^{Sey/Sey}$) causes severe forebrain abnormalities, demonstrating the important role that *Pax6* plays in the development of this structure (Schmahl et al., 1993). Studies conducted over the last two decades have shown that *Pax6* is involved in a variety of different developmental processes that are vital for the generation of the forebrain. These include neuronal patterning, proliferation, migration and axon guidance (Mastick et al., 1997; Quinn et al., 2007; Stoykova et al., 2000; Talamillo et al., 2003).

1.3.2 *Pax6* expression in the developing mouse forebrain

Pax6 expression in the mouse begins at around E8.5. At this stage *Pax6* is expressed throughout the neuroepithelium that will form the hindbrain, the forebrain and the eye (Walther and Gruss, 1991). By E9.5 *Pax6* expression is down regulated in the hindbrain, but is still found throughout the structures of the forebrain (Fig. 5A) (Mastick et al., 1997). As neurogenesis begins at around E10.5 postmitotic neurons downregulate *Pax6,* but expression is maintained in the proliferative populations of cells at the ventricular zones of the diencephalon and telencephalon. Some postmitotic neurons within the prethalamus and ventral telencephalon also maintain *Pax6* expression after exiting the cell cycle. By E12.5 *Pax6* expressing cells can be seen at the ventricular zone of the developing cortex and at lower levels in the ventral telencephalon. The level of expression is not uniform but is instead arranged in a rostro-lateral^{high} to caudo-medial^{low} gradient. *Pax6* is also expressed by a stream of postmitotic cells from the PSPB to the amygdaloid region. Within the diencephalon *Pax6* expression is downregulated in the postmitotic cells of the thalamus but is maintained through the depth of the prethalamus (Fig 5B). At the later stage of E14.5 *Pax6* expression has largely disappeared from the ventricular zone of the ventral telencephalon with the exception of the region adjacent to the PSPB. In the diencephalon *Pax6* has been further downregulated and is now only found at the upper and lower edges of the prethalamus, within the epithalamus and at the ventricular zone (Fig. 5C). After this Pax6 is further downregulated so that by E17.5 expression of the gene is only present within proliferative cells of the cortex, a thin strip of cells at the upper edge of the prethalamus and at the epithalamus (Fig. 5D). In the adult *Pax6* is only expressed by a small number of cells within the prethalamus and the hippocampus (Nacher et al., 2005).

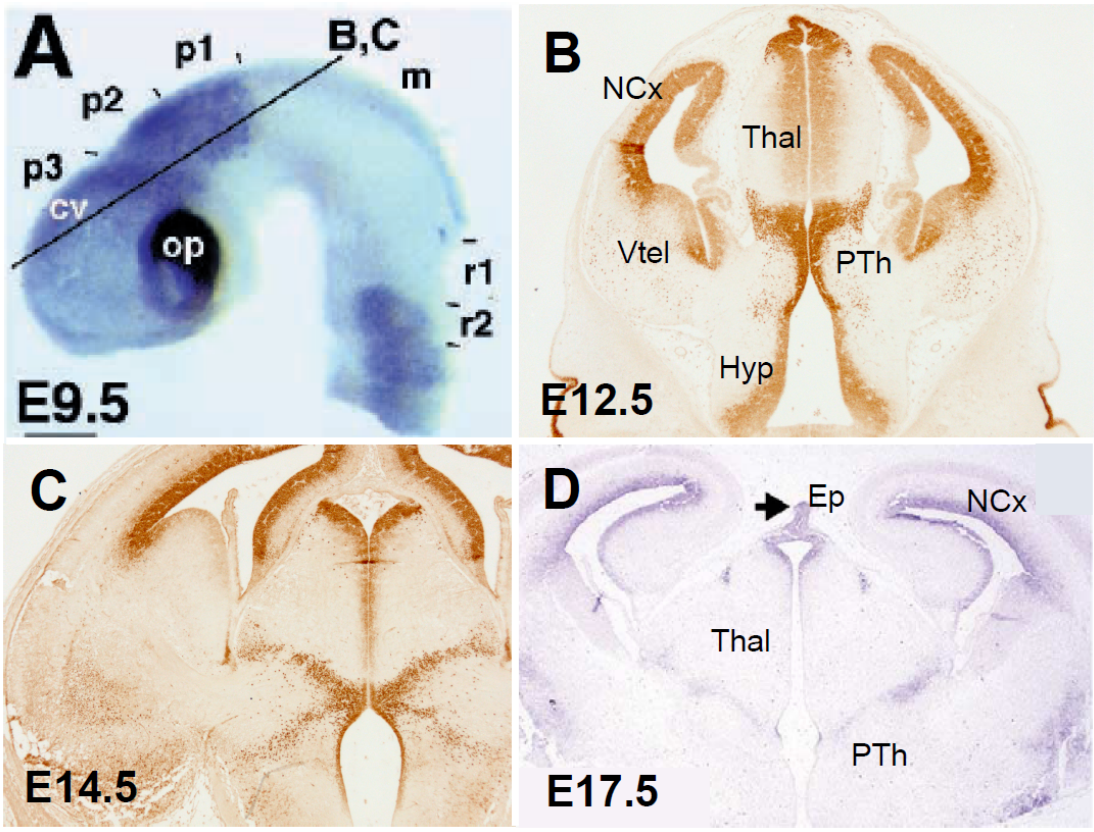


Figure. 5. *Pax6* expression during embryonic mouse development. (A) Whole mount *in-situ* hybridisation for *Pax6* at E9.5 showing *Pax6* expression throughout the forebrain and optic vesicle (from Mastick *et al*, 1997). **(B, C)** Immunohistochemistry for *Pax6* showing expression in coronal sections through the forebrain at E12.5 (B) and E14.5 (C). **(D)** *In-situ* hybridisation for *Pax6* at 17.5 (from Estivill-Torrus *et al*, 2001). Abbreviations: Op, optic vesicle; Hyp, hypothalamus; NCx, neocortex; Vtel, ventral telencephalon; PTh, prethalamus; Thal, Thalamus; Ep, epithalamus.

1.3.3 The role of *Pax6* in the patterning of the telencephalon.

An important step in the development of the telencephalon is its division into the dorsal telencephalon, or pallium, and the ventral telencephalon, or subpallium. The pallium will form the cortex while the subpallium will give rise to the striatum and the basal ganglia. The two structures are separated by the PSPB (sometimes known as the cortico-striatal boundary) which forms at around E12.5. This is a physical boundary which consists of a glial fascicle and is also an important gene expression boundary separating dorsal and ventral domains.

The specification of the cells along the dorso-ventral axis of the telencephalon involves a number of different transcription factors. Genes such as *Mash1* and *Gsh2* promote ventral telencephalic cell fates while others such as *Ngn2* and *Pax6* promote dorsal cell fates. Studies of mice deficient for these genes have demonstrated the important role they play in the specification of dorso-ventral character (Parras et al., 2002; Yun et al., 2001). In $Pax6^{Sey/Sey}$ embryos the telencephalon becomes ventralised. The expression domain of molecular marker genes for ventral telencephalic cells (such as *Dlx2* and *Mash1*) extend much further dorsally than normal while dorsal markers (such as *Emx1* and *Ngn2*) are retracted dorsally. This ventralisation leads to the respecification of dorsal telencephalic cells which adopt a character similar to that of the LGE (Stoykova et al., 1996; Stoykova et al., 2000). Conversely in mutant mice deficient in ventrally expressed transcription factor *Gsh2* the telencephalon undergoes dorsalisation; with dorsally expressed markers extending further ventrally. When both *Gsh2* and *Pax6* are lost the expression domain of dorsal and ventral markers is normal (Toresson et al., 2000). This suggests that *Pax6* and *Gsh2* act in a mutually repressive manner in order to generate dorsal and ventral cell types in the correct position.

Pax6 and *Gsh2* are also particularly important for the formation of the PSPB which forms the boundary between the dorsal pallium and the ventral subpallium. At E10.5 before the PSPB forms *Pax6* is expressed throughout the dorsal telencephalon and *Gsh2* is expressed ventrally, the expression domains of the two genes overlap within the LGE with a subset of cells expressing both genes. By E11.5 the boundary between the two cellular domains becomes sharper and more highly defined (Corbin et al., 2003; Yun et al., 2001). A recent study has provided evidence that this

refinement of the boundary between the *Gsh2* and *Pax6* expressing regions can occur by two different processes. Firstly, cells can migrate to a position where they are surrounded by other cells expressing the same gene, and secondly some cells change their gene expression in order to match that of the cells surrounding them (Cocas et al., 2011). The sorting of cells to form the sharp boundary is thought to be at least in part due to the different adhesive properties of cells at either side of the PSPB. Cells within the dorsal telencephalon express cell adhesion molecule R-cadherin, while ventral telencephalic cells do not causing a difference in adhesive properties. R-cadherin expression is regulated by *Pax6* and in $Pax6^{Sey/Sey}$ embryos, which lack functional *Pax6*; the expression of *R-cadherin* is greatly reduced. In these embryos the PSPB does not form correctly; the glial fascicle is not present and the sharp gene expression boundary between the ventral and dorsal domains is lost (Stoykova et al., 1997).

The vast majority of cortical GABAergic interneurones are not generated at the ventricular zone of the cortex but are in fact derived from the ganglionic eminences of the subpallium (Marin and Rubenstein, 2001). In $Pax6^{Sey/Sey}$ embryos GABAergic interneurons form subpial ectopias at the surface of the cortex which express *Dlx2* and *ErbB4*; marker genes of subpallium derived interneurons. Fate mapping experiments have shown however, that these ectopic interneurones do not originate from the subpallium, but instead are derived from the *Emx1* lineage cells which would normally give rise to glutamatergic cortical neurons (Kroll and O'Leary, 2005). This change in cell fate from dorsal/glutamatergic fate to ventral/GABAergic fate in the absence of *Pax6* further demonstrates the vital role that the *Pax6* plays in the specification of the dorsal telencephalon.

1.3.4 *Pax6* and the arealisation of the cortex

Across its surface the cortex can be divided into areas that differ from each other in their function and the axonal connections they establish. For example the primary visual cortex (area V1) is located at the caudal-most part of the cortex and forms thalamocortical conections with the dLGN while the somatosensory cortex (S1) is located rostral to V1 and forms connections with VB. Several transcription

factors are expressed in a gradient across the cortex, *Pax6* is expressed across the progenitor cells of the cortex in a rostro-lateral^{high} to caudo-medial^{low} gradient and it is thought that this expression gradient has an important role in specifying the different cortical areas. In *Pax6*$^{Sey/Sey}$ embryos the arealisation of the developing cortex has been analysed by looking at the expression pattern of genes specific to certain areas. It was found that molecular markers for caudomedial areas such as V1 were expanded while markers for rostrolateral areas like the primary motor cortex (M1) were reduced. This suggests that *Pax6* is required for normal arealisation of the cortex (Bishop et al., 2002). The thalamocortical tract does not form in these embryos so it is impossible to test if this shift in marker expression correlates with altered axonal connections. Another study using conditional mutagenesis to specifically knockout *Pax6* expression in the cortex found a similar effect to that seen in the *Pax6*$^{Sey/Sey}$ embryos with a caudomedial shift in rostrolateral area markers. This shift however was much less pronounced than that seen in the *Pax6*$^{Sey/Sey}$ embryo. Despite this change the topography of thalamocortical connections to the cortex appears unchanged (Pinon et al., 2008).

The mouse models discussed so far have looked at the effect of removing *Pax6*. In a mouse model where *Pax6* is overexpressed one might predict the opposite effect to that seen in the loss of function mutants, with rostrolateral area markers ectopically extending caudomedially. The *Pax77* transgenic mouse model carries several copies of the human *Pax6* locus, which causes an increase in *Pax6* protein levels. Despite this increase in *Pax6* expresion the arrangement of area markers in *Pax77* embryos is unchanged, as is the topography of thalalmocortical connections (Manuel et al., 2007). Though the global level of *Pax6* protein is increased in the *Pax77* telencephalon, the gradient of expression across the cortical progenitors is maintained. The fact that we see no change in arealisation of the cortex in these mice suggests that it is the relative level of *Pax6* expression along the rostrolatera-caudomedial gradient that is particularly important for area identity rather than total level of *Pax6*.

1.3.5 The role of *Pax6* in the patterning of the diencephalon

The study of loss of function mutant mice has demonstrated that *Pax6* plays a vital role in the regionalisation of the telencephalon. These mice have also provided evidence that *Pax6* regulates the molecular patterning of the diencephalon. At E9.5 *Pax6* is expressed throughout the diencephalon but at later stages *Pax6* expression is restricted to the epithalamus, the prethalamus and the ventricular zone of the thalamus. The developing diencephalon can be divided into prosomeres P1, P2 and P3 according to the different marker genes these regions express (Puelles and Rubenstein, 2003). In $Pax6^{Sey/Sey}$ embryos the expression patterns of these marker genes is altered. The expression of markers of the thalamus (P2) such as *VMAT2* and *Hbnf* appear reduced, and are retracted dorsally, while the expression pattern of prethalamus (P3) markers such as *Dlx2* are expanded dorsally (Pratt et al., 2000b; Warren and Price, 1997). This suggests that *Pax6* is required for the normal specification of the thalamus and prethalamus. The ZLI forms the boundary between the prethalamus and the thalamus, and expresses the morphogen *Shh* which is known to be important for the patterning of both the thalamus and the prethalamus (Vue et al., 2009). In $Pax6^{Sey/Sey}$ embryos the *Shh* expression domain within the ZLI is expanded, as is the expression domain of *Nkx2.2* which is normally expressed immediately above and below the ZLI (Grindley et al., 1997; Pratt et al., 2000b). This apparent expansion in the size of the ZLI may be an important cause of the patterning defects observed in $Pax6^{Sey/Sey}$ embryos and raises the possibility that *Pax6* influences the patterning of the diencephalon by regulating the expression of *Shh* at the ZLI.

1.3.6 The role of *Pax6* in neuronal migration

The formation of the brain requires the generation of many different cell types. During development, some of these cells have to migrate from the region where they were born to occupy specific locations where they are functionally required. In $Pax6^{Sey/Sey}$ embryos the PSPB fails to form, as has been discussed above, and the rate of migration from the subpallium to the pallium is increased (Chapouton

et al., 1999). This increase in migration suggests that *Pax6* acts to restrict ventro-dorsal migration, by controlling the development of the PSPB.

The migration of cortical neurones from the proliferative ventricular zone of the developing cortex to positions within the cortical plate is an important process for the formation of the cortical layers. By E16.5 the ventricular zone (VZ) in *Pax6*$^{Sey/Sey}$ embryos is greatly thickened when compared to WT embryos, while the thickness of the cortical plate is reduced (Schmahl et al., 1993). Neuronal labelling experiments using BrdU have shown that the ability of late born neurons to migrate from the ventricular zone and subventricular zone (SVZ) into the cortical plate is severely reduced in *Pax6*$^{Sey/Sey}$ embryos; however early-born neurons are able to migrate normally (Caric et al., 1997). This may be a factor in causing the accumulation of cells in at the VZ and SVZ of embryos deficient in *Pax6*. Transplant experiments showed that when *Pax6*$^{Sey/Sey}$ cells were injected into WT rat cortices the late-born *Pax6*$^{Sey/Sey}$ cells were able to migrate into the cortex with the same efficiency as injected WT cells (Caric et al., 1997). This suggests that cortical cells do not require *Pax6* expression in order to migrate into the cortical plate but rather *Pax6* acts to set up the environment required for this migration. In chimeric embryos which contain a mixture of *Pax6*$^{Sey/Sey}$ and *Pax6*$^{+/+}$ cells, the *Pax6*$^{Sey/Sey}$ cells are largely restricted to the VZ and SVZ and have a reduced ability to cross the intermediate zone in order to reach the cortical plate (Talamillo et al., 2003). Contrary to the transplant experiments of Caric et al, (1997) this result suggests that there is in fact a cell autonomous requirement for *Pax6* expression for the migration of neurons into the cortical plate. It is likely that *Pax6* functions both in a cell-autonomous and non cell-autonomous fashion to influence cortical migration

1.3.7 The role of *Pax6* in cortical neurogenesis

For the complex neuronal circuits of the mammalian forebrain to form, the numerous neuronal cell types within the cortex must be generated during development. These different cell types must also adopt their correct position within the layered structure of the cortex. The excitatory neurons of the cortex are born during corticogenesis at the proliferative regions of the VZ and SZV, while

GABAergic interneurons are produced within the ventral telencephalon and migrate into the cortex (Marin and Rubenstein, 2001). Cortical neurons originate at the VZ/SVZ from radial glial cells (RGCs), neuronal precursors with processes which span the depth of the developing cortex (Malatesta et al., 2000). This occurs in one of two ways: direct neurogenesis and indirect neurogenesis. During direct neurogenesis the RGCs, or apical progenitor cells (APCs) divide at the VZ producing a postmitotic neuron which migrates along the radial glial process into the cortical plate (Noctor et al., 2001). During indirect neurogenesis APCs divide to produce a basal progenitor cell (BPC) which migrates to the SVZ where it divides again to give two postmitotic neurons which then migrate into the cortical plate (Miyata et al., 2004). *Pax6* is expressed by APCs but is downregulated in BPCs and postmitotic neurons (Englund et al., 2005). In $Pax6^{Sey/Sey}$ embryos RGCs show reduced neurogenic potential, which is consistent with the observation of the reduced thickness of the cortical plate (Heins et al., 2002). Conversely *Pax6* overexpression causes an increase in cortical neurogenesis (Sansom et al., 2009). In $Pax6^{+/+} \leftrightarrow Pax6^{Sey/Sey}$ chimeras, $Pax6^{Sey/Sey}$ cells are underrepresented in the cortical plate compared to $Pax6^{+/+}$ cells, and there is evidence that this is due to a reduction in the pool of progenitor cells at the VZ caused by premature cell cycle exit in APCs which lack *Pax6* (Quinn et al., 2007). This reduction in progenitor cells may explain the reduced neurogenic potential of RGCs observed by Heins et al (2002), and is consistent with a role for *Pax6* in regulating neurogenesis in the cortex by controlling the rate of cell cycle exit by APCs. BPCs are thought to be particularly important for the production of the superficial cortical layers (Tarabykin et al., 2001). There is evidence that *Pax6* expression is cell autonomously required for the promotion of BPC production (Quinn et al., 2007). *Pax6* may specify BPC cell fate by directly promoting the expression of BPC specific transcription factors such as *Tbr2* (Englund et al., 2005).

1.3.8 *Pax6* and forebrain axon guidance.

In order for the forebrain to function normally axonal connections must form between different forebrain regions. Over the last two decades a number of different

genes and cellular interactions have been shown to be important for the guidance of growing axons within the forebrain. It is unclear however how the process of axon guidance is controlled and regulated. One possibility is that transcription factors control axon guidance within the forebrain by regulating the expression of key axon guidance genes. Mutant mice with loss of function mutations for a number of different transcription factors have been shown to have a wide range of axon pathfinding defects which is consistent with the idea that transcription factors exert some control over axon guidance during development (Lopez-Bendito and Molnar, 2003).

Pax6 has been shown to be required for the formation of several major axon tracts during forebrain development. The thalamocortical tract which connects the nuclei of the thalamus with the cortex completely fails to form in *Pax6*$^{Sey/Sey}$ embryos (Pratt et al., 2002) (see section 1.4 for greater depth). The corpus callosum is a large mammal-specific axon tract linking the two cerebral hemispheres. In adult mice where *Pax6* expression has been specifically reduced in progenitor cells of the cortex during neurogenesis, callosal axons are disorganised and fail to form connections with the opposite hemisphere (Boretius et al., 2009). The tract of the postoptic commissure (TPOC) begins to form in the mouse at E9.5 when the first axons extend from cells close to the optic stalk and grow through the diencephalon, reaching the thalamus by E11.5. In embryos that lack *Pax6,* these axons show severe pathfinding errors, particularly within the prethalamus where *Pax6* would normally be highly expressed (Mastick et al., 1997). Expression of the cell adhesion molecule *R-cadherin* is regulated by *Pax6* (Stoykova et al., 1997), in WT embryos *R-cadherin* is expressed at the prethalamus; while in *Pax6*$^{Sey/Sey}$ embryos this expression is lost. When R-cadherin expression is reintroduced at the prethalamus by electroporation the TOPC axon pathfinding defect in *Pax6*$^{Sey/Sey}$ embryos is rescued. This demonstrates that *Pax6* influences TPOC axon guidance by regulating the expression of *R-cadherin* which promote axon growth through the prethalamus (Andrews and Mastick, 2003). In addition to cell adhesion molecules, *Pax6* may also regulate the expression of diffusible guidance cues. In *Pax6*$^{Sey/Sey}$ embryos the expression of attractive *Semaphorins* are reduced in the cortex while in the thalamus there is evidence that the expression of *Netrin1* is upregulated (Jones et al., 2002; Tsuchiya et

al., 2009). This suggests that *Pax6* can control axon guidance by regulating the expression of certain axon guidance cues.

1.4 The Role of *Pax6* in the development of the thalamocortical tract

1.4.1 Overview

The thalamocortical tract is a large axon tract that connects the nuclei of the thalamus to the cortex and is important for the relaying of information from the periphery to the cortex. In the mouse the thalamocortical tract forms between E12.5 and E18.5 (Auladell et al., 2000). Research conducted over the last 20 years has identified a number of mechanisms by which thalamocortical axons (TCAs) are guided to their target areas within the cortex. Axon guidance cues expressed by different tissues along the route of the tract can direct TCA growth, one example being *Slit* proteins that are expressed at the hypothalamus and repel TCAs from this region causing them to turn towards the telencephalon (Bagri et al., 2002). In addition pioneer axon populations originating from the prethalamus and internal capsule zone, and 'guidepost cells' within the ventral telencephalon provide further guidance information to navigating TCAs (Lopez-Bendito et al., 2006; Molnar and Cordery, 1999). Despite this understanding of a number of TCA guidance mechanisms, very little is known about how the different axon guidance genes and cellular processes are controlled. It is likely that transcription factors exert control over axon guidance by regulating the expression of key genes involved in TCA guidance.

Transcription factor *Pax6* is known to be involved in a variety of important developmental processes required for the formation of the forebrain, including axon guidance (Mastick et al., 1997). The mature neurons of the thalamus which extend TCAs express *Pax6* at the progenitor cell stage. *Pax6* is also expressed in several populations of cells along the route of the thalamocortical tract, at a time when TCAs are navigating towards the cortex (Fig. 6A, B) (Walther and Gruss, 1991) .In addition *Pax6* may be expressed by some cells that extend pioneer axons or by 'guidepost cells' within the ventral telencephalon; either at the progenitor cell stage or as mature neurons. This means that *Pax6* is expressed in the correct place to regulate the expression of diffusible axon guidance cues and their receptors on the growth cone.

Pax6 is also in a position to influence the formation of pioneer axon tracts and guidepost cells.

In *Pax6*$^{Sey/Sey}$ embryos the thalamocortical tract does not form, with TCAs unable to reach the cortex (Jones et al., 2002; Kawano et al., 1999; Pratt et al., 2002). This suggests that *Pax6* is required for the guidance of TCAs, but it remains unclear exactly how *Pax6* is influencing TCA pathfinding. The aim of this thesis is to examine this question and determine some of the mechanisms by which *Pax6* controls the development of the thalamocortical tract.

1.4.2 Transcription factors and thalamocortical axon guidance

The study of transgenic mice with loss of function mutations for a number of axon guidance cues, such as *Netrin1, Slit2* and *Sema6A*, has demonstrated the role that these genes play in the guidance of TCAs to the cortex (Bagri et al., 2002; Braisted et al., 2000; Little et al., 2009). Loss of function mutants for several developmentally important transcription factors such as *Dlx2, Mash1, Emx2, Pax6* and *Ebf1* also display profound TCA pathfinding abnormalities. *Mash1* is expressed by progenitor cells at the ventricular zone of the developing prethalamus and ventral telencephalon. In *Mash1* deficient mice TCAs do not reach the telencephalon but instead either form aberrant bundles within the thalamus or course ventrally along the diencephalic side of the DTB (Tuttle et al., 1999). In *Emx2* deficient mice TCA guidance is also disturbed. Unlike in *Mash1*$^{-/-}$ embryos TCAs do reach the telencephalon but are abnormally deflected ventrally within the ventral telencephalon and do not cross the PSPB to reach the cortex (Lopez-Bendito et al., 2002). Interestingly, in both of these transgenic mice the cells which extend pioneer axons from the prethalamus and internal capsule zone are either absent or displaced. This suggests that these transcription factors are required for the normal development of the pioneer axon tracts important for TCA guidance. In *Mash1*$^{-/-}$ embryos the migration of guidepost cells from the LGE to the MGE fails to occur. This migration is required for the guidance of TCAs through the ventral telencephalon and may account for the inability of TCAs to cross the DTB in these embryos (Lopez-Bendito et al., 2006).

Disruption in the expression of axon guidance cues can disturb TCA guidance. In *Mash1*$^{-/-}$ embryos the expression of guidance cue *Netrin1* is downregulated within the ventral telencephalon (Tuttle et al., 1999). In embryos deficient for transcription factor *Ebf1* TCAs reach the cortex but are abnormally deflected ventrally. In these embryos the expression of axon guidance cues *Netrin1* and *Sema6A* is disrupted, which may account for the TCA guidance errors seen in the ventral telencephalon (Garel et al., 2002). This provides further evidence that transcription factors can influence thalamocortical development by regulating the expression of axon guidance cues along the route of the tract.

1.4.3 The thalamocortical tract in *Pax6* deficient embryos

Several studies have examined how the thalamocortical tract develops in full *Pax6* loss of function mice and rats. The first study to address this question used axon tract tracing and immunohistochemistry to examine the morphology of the thalamocortical tract in embryonic *Pax6*$^{Sey/Sey}$ rats. Kawano et al (1999) found that in *Pax6*$^{Sey/Sey}$ embryos TCAs do not reach the cortex as in the WT. Instead TCAs extend ventrally within the diencephalon in the direction of the hypothalamus, rather than turning laterally to enter the telencephalon. They also identified a large axon bundle, not present in the WT, which appeared to emanate from the ventral aspect of the ventral telencephalon. This axon bundle was presumed to be made up of TCAs which have crossed the DTB to reach the telencephalon (Fig. 6B, C). Later studies have also shown that TCAs extend towards the hypothalamus in embryos which lack *Pax6* (Hevner et al., 2002; Jones et al., 2002; Pratt et al., 2002). This is despite the fact that the hypothalamus is normally repulsive to TCAs (Braisted et al., 1999). The repulsive character of the hypothalamus is mediated by signalling between diffusible *Slit* proteins and their receptors on the growth cone, *Robo* (Bagri et al., 2002; Lopez-Bendito et al., 2007). This raises the possibility that the expression of *Slits* or *Robos* is altered in *Pax6* deficient embryos, this question will be addressed in chapter three. The identity of the axon bundle present in the *Pax6*$^{Sey/Sey}$ ventral telencephalon is less clear however. It has been observed by immunohistochemistry for axonal markers in several studies, but tract tracing has failed to determine conclusively that its origin is within the thalamus. This ambiguity is also addressed in chapter three.

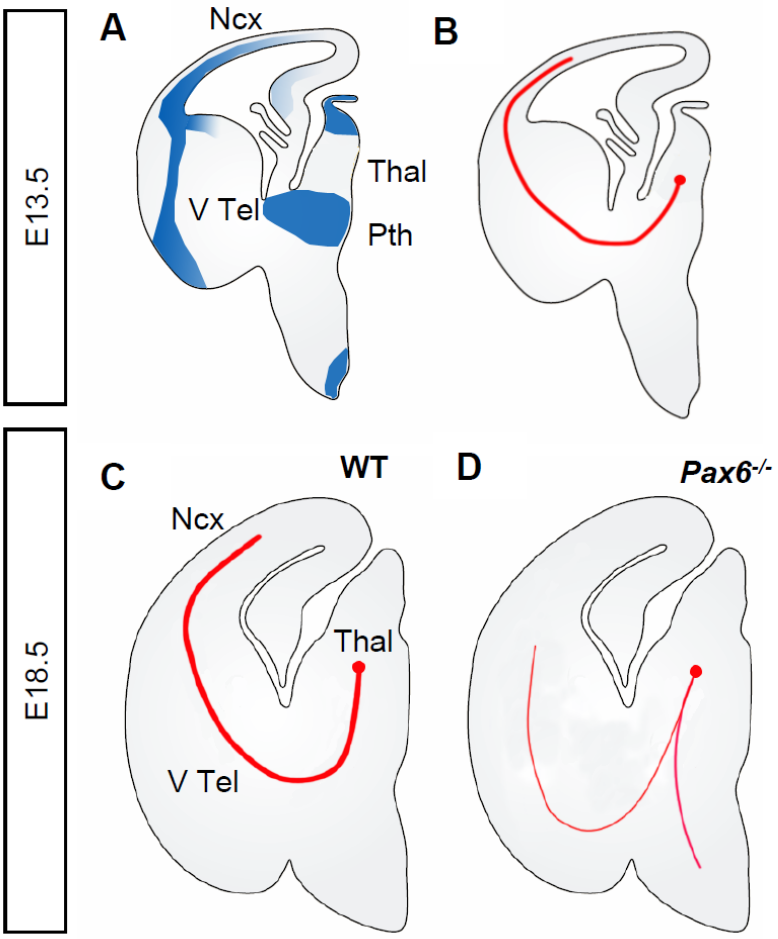


Figure 6. *Pax6* and the thalamocortical tract. (A) Schematic diagram of a coronal section through an E13.5 mouse forebrain indicating where *Pax6* is expressed (blue). **(B)** Schematic showing the route of the thalamocortical tract (red line) which comes into contact with *Pax6* expressing cells at several positions along this route. **(C)** Schematic representing the thalamocortical tract at E18.5 in WT embryos. **(D)** The thalamocortical tract in *Pax6* loss of function mutant embryos according to Kawano et al, 1999, Hevner et al, 2002 and Jones et al, 2002. TCAs aberrantly enter the hypothalamus and TCAs that reach the ventral telencephalon cannot cross the PSPB to reach the cortex. Abbreviations: Ncx, neocortex; Pth, prethalamus; Thal, thalamus; Vtel, ventral telencephalon.

1.4.4 The cell-autonomous and non cell-autonomous role for *Pax6* in thalamocortical axon guidance.

We have seen that *Pax6* expression is required for the correct formation of the thalamocortical tract, but this presents the question, where exactly is *Pax6* function required? There are two possibilities; firstly that *Pax6* expression is required within the cells of the thalamus that extend TCAs (cell-autonomously) and acts to 'programme' TCAs to respond to guidance signals present in the forebrain, or secondly that *Pax6* expression is needed in cells along the route of the tract (non-cell-autonomously) and regulates axon guidance mechanisms that guide TCAs. It is equally possible that *Pax6* is required both cell autonomously and non-cell-autonomously. Several studies have attempted to examine this question. Pratt et al (2000) used an organotypic co-culture technique to examine how $Pax6^{Sey/Sey}$ TCAs behave when confronted with WT ventral telencephalon and how WT TCAs behave when confronted by $Pax6^{Sey/Sey}$ ventral telencephalon. Their analysis showed that when WT thalamus and ventral telencephalon were cultured in contact TCAs were able to enter the ventral telencephalon normally and make a dorsal turn reminiscent of that seen *in vivo*. When $Pax6^{Sey/Sey}$ thalamus was cultured with WT ventral telencephalon, $Pax6^{Sey/Sey}$ TCAs were able to enter the ventral telencephalon but did not proceed as far as WT TCAs and did not make a dorsal turn. This inability of $Pax6^{Sey/Sey}$ TCAs to respond to guidance signals present within the ventral telencephalon suggests that *Pax6* expression is required in thalamic cells for TCA guidance. When WT thalamus was cultured with $Pax6^{Sey/Sey}$ ventral telencephalon, WT TCAs entered the ventral telencephalon but did not turn dorsally as they did when growing into WT tissue. This suggests that the guidance signals present at the ventral telencephalon are disrupted in $Pax6^{Sey/Sey}$ embryos, and that *Pax6* expression is required at the ventral telencephalon for the guidance of TCAs to the cortex. Taken together the data presented in this study suggest that for the thalamocortical tract to develop normally, *Pax6* expression is required both in thalamic cells that extend TCAs and in other cells along the route of the tract. This means that *Pax6* must play a role in the regulation of molecular guidance cues or other guidance mechanisms at the growth cone and in other positions such as the prethalamus, the hypothalamus or the ventral telencephalon. Jones et al (2002) showed that the expression of attractive

Semaphorins, Sema3C and *Sema5A,* are reduced in the cortex of *Pax6* deficient mice which demonstrates that *Pax6* may regulate the expression of guidance cues required for TCA guidance. *Netrin1* expression appears normal in $Pax6^{Sey/Sey}$ ventral telencephalon (Pratt et al., 2000b) but other molecular guidance cues and guidance mechanisms are present within this region, and they may be affected by the loss of *Pax6*.

Another approach used to explore this question is conditional mutagenesis. By using the cre-lox system it is possible to knockout target gene expression in specific cell types or at specific time points during development. Using this technique two studies have examined how the development of the thalamocortical tract is affected by the deletion of *Pax6* expression in different cell populations along the route of the tract. Pinon et al (2008) used an $Emx1^{Cre}$ allele to delete *Pax6* expression within the cortex; they showed that in these embryos TCAs are able to reach the cortex normally. They also observed that the topographic arrangement of the thalamocortical projections is maintained despite the loss of *Pax6* in the cortex. This study shows that *Pax6* expression is not required at the cortex for the thalamocortical tract to develop normally, and therefore, that *Pax6* must be influencing TCA guidance in a different population of cells. Another study (Simpson et al., 2009) used a $Six3^{Cre}$ allele to drive *Pax6* deletion in a subpopulation of *Pax6* expressing cells found close to the amygdaloid region in the developing ventral telencephalon. The co-culture analysis of Pratt et al, 2000 had shown that *Pax6* expression is required within the ventral telencephalon for the normal TCA guidance. This study aimed to determine if *Pax6* function was specifically required in this *Six3* expressing population of cells in order to guide TCAs through the ventral telencephalon. Tract tracing experiments revealed that a large number of TCAs are able to reach the cortex in conditional mutant embryos. Some TCAs do not reach the cortex, however, and either do not cross the DTB to reach the telencephalon or aberrantly project ventrally within the ventral telencephalon. This confirms that indeed *Pax6* is required in these cells for the guidance of TCAs. An important axon guidance mechanism in the ventral telencephalon is the tangential migration of *Islet1* expressing ‘guidepost cells’ to form a permissive ‘corridor’ through which TCA can grow (Lopez-Bendito et al., 2006). In conditional mutant embryos the migration of

these cells is disrupted, with the corridor becoming abnormally widened, which may account for the TCA pathfinding errors seen in this region. This suggests that one way in which *Pax6* influences TCA guidance within the ventral telencephalon is to control the migration of these cells, restricting them into a tight corridor in order to effectively guide the thalamic axons.

The evidence from the above studies, which have examined thalamocortical development in different *Pax6* loss of function mutant embryos, seem to confirm that *Pax6* is required both in the progenitors of thalamic neurons which extend TCAs and in other cell populations along the route of the tract. It is still unclear however exactly how *Pax6* regulates TCA guidance in these cells.

1.5 Aims

The aim of this thesis is to examine the way in which *Pax6* controls the development of the thalamocortical tract. In particular I intend to investigate the role that *Pax6* plays in the regulation of guidance molecules and other guidance mechanisms which direct TCAs. Using different mutant mouse models I will examine how *Pax6* expression within cells of the thalamus and cells along the route of the tract influence thalamocortical development.

Chapter 2: Materials and Methods

2.1 Animal use and breeding

Pax6$^{Sey/+}$ mice were maintained on a CD-1 background by crossing *Pax6*$^{Sey/+}$ males with WT CD-1 females. All *Pax6*$^{Sey/Sey}$ and WT (*Pax6*$^{+/+}$) control embryos were obtained from crosses of heterozygous (*Pax6*$^{Sey/+}$) mice. Genotyping of these mice was accomplished by observation of the 'small eye' phenotype displayed by heterozygous mice (Hogan et al., 1988). The eye phenotype of the embryos generated by heterozygous crosses was also used to distinguish between WT (*Pax6*$^{+/+}$), heterozygous (*Pax6*$^{Sey/+}$) and homozygous (*Pax6*$^{Sey/Sey}$) embryos (Hill et al., 1991).

To generate conditional *Pax6* knockout embryos floxed *Pax6* mutant mice (*Pax6*$^{loxP/loxP}$) (Simpson et al., 2009) were crossed with mice carrying the *Gsh2Cre* allele, which express Cre recombinase under the influence of the *Gsh2* promoter (Kessaris et al., 2006). *Pax6*$^{loxP/+}$, *Gsh2Cre*$^{+/-}$ mice were maintained on a CD-1 background by crossing transgenic males with WT CD-1 females. To enable the detection of Cre activity, *Pax6*$^{loxP/loxP}$ mice were crossed with mice RCE:LoxP mice (Sousa et al., 2009). The cells of RCE:LoxP reporter mice cells show strong EGFP expression in the presence of Cre recombinase, these mice were created by the insertion of a floxed neo cassette upsteam of an EGFP coding sequence within the *Rosa26* locus (Sousa et al., 2009). *Pax6*$^{loxP/+}$, RCE$^{+/+}$mice were then maintained on a CD-1 background by crossing transgenic males with WT CD-1 females. To obtain embryos *Pax6*$^{loxP/+}$, RCE$^{+/+}$ males were crossed with *Pax6*$^{loxP/+}$, *Gsh2Cre*$^{+/-}$ females. *Pax6*$^{loxP/loxP}$, *Gsh2Cre*$^{+/-}$, RCE$^{+/-}$ embryos were considered conditional knockouts (designated *Pax6*^{cKO}) while *Pax6*$^{+/+}$, *Gsh2Cre*$^{+/-}$, RCE$^{+/-}$ embryos were used as controls.

To generate DTy54/*Pax6*^{cKO} embryos floxed *Pax6* mice were crossed with DTy54 mice. The DTy54 mouse expresses tau-GFP under the influence of the entire human *Pax6* regulatory elements. This transgenic mouse was created by the insertion of a tau-GFP reporter cassette into the translation start site of exon 4 of the *Pax6* gene contained on a yeast artificial chromosome (YAC). The YAC was then integrated into the genome following microinjection of one-cell embryos. The resulting mice express tau-GFP in cells which would normally express *Pax6* (Tyas et al., 2006). *Pax6*$^{loxP/+}$, DTy54$^{+/-}$ males were then crossed with *Pax6*$^{loxP/+}$, *Gsh2Cre*$^{+/-}$

females to obtain embryos. *Pax6*$^{loxP/loxP}$, *Gsh2Cre*$^{+/-}$, DTy54$^{+/-}$ embryos were considered conditional knockouts while *Pax6*$^{+/+}$, *Gsh2Cre*$^{+/-}$, DTy54$^{+/-}$ embryos were used as controls. It should be noted that the DTy54/*Pax6*^{cKO} embryos generated for this experiment did not contain a Cre recombinase reporter allele, and that any GFP expression is a result of the DTy54 tau-GFP reporter not Cre recombinase expression.

To obtain *Pax6*$^{+/+}$↔*Pax6*$^{Sey/Sey}$ chimeric embryos, *Pax6*$^{Sey/Sey}$ embryonic stem cells which carried one copy of the TP6.3 tau-GFP transgene (Pratt et al., 2000a) were injected into blastocycts from C57BL/6 x CBA crosses. Blastocysts were then transferred to the uterus of pseudo-pregnant females and were allowed to develop to the desired developmental stage. Resulting chimeric embryos express tau-GFP in all cells which originate *Pax6*$^{Sey/Sey}$ embryonic stem cells regardless of whether the cells express *Pax6*, cells not expressing tau-GFP originate from *Pax6*$^{+/+}$ embryonic stem cells. Maintenance of embryonic stem cells, blastocyst injection and blastocyst transfer were carried out by Michael Molinek and Isabel Martin.

All the mice used in this study were maintained in accordance with Home Office and University of Edinburgh animal welfare guidelines.

2.2 Embryo collection and histology

For the staging of embryos midday on the day of vaginal plug detection was considered as embryonic day 0.5 (E0.5). Pregnant females were culled either by an overdose of anaesthetic or cervical dislocation. Embryos were then harvested from the females into ice cold phosphate buffered saline (PBS) and tissue samples were removed from the front paw for genotyping analysis by PCR. For embryos aged E12.5 to E15.5 heads were removed and fixed in 4% paraformaldahyde (PFA) in PBS overnight at 4^{o}C, with shaking. For embryos aged E16.5 to E18.5 the whole brain was removed and then fixed as above. Following fixation the heads/brains were then washed with PBS and either dehydrated and embedded in paraffin wax or embedded in 4% agarose. Paraffin wax embedded heads/brains were sectioned coronally at 10µm using a microtome. Sections were then mounted on Superfrost Plus slides (VWR) and stored until use at 4^{o}C. Agarose embedded brains were

sectioned coronally or sagittally at 100µm using a vibratome (Leica VT1000S). Sections were collected in PBS in 24 well plates and stored until use at 4°C.

2.3 Genotyping

Genomic DNA was extracted from ear notches of mice or paw samples of embryos by applying 75µl lysis buffer (25mM NaOH, 0.2mM EDTA) and heating to 90°C for 30 minutes followed by cooling to 4°C, after which the solution was neutralised by addition of 75µl 40mM Tris HCl. PCR was then used to amplify DNA fragments from the genomic DNA samples; the composition of the reaction mixture was as follows.

- 2.5µl dNTPs (each at 2.5mM, Fermentas)
- 5µl 5 x 'GoTaq' reaction buffer (Promega)
- 0.5µl Primers (each at 25M)
- 0.2µl 'GoTaq' DNA polymerase (Promega)
- 15.8µl H2O
- 1µl DNA sample.

For amplification the following programme was used: 94°C for 4 minutes followed by a sequence of 94 °C for 30 seconds 62 °C for 45 seconds and 72 °C for 1 minute, this sequence was then repeated 33 times. After this the reaction mixture was held at 72 °C for 10 minutes and 10 °C for 10 minutes. To separate the amplified DNA fragments 10µl of reaction mixture was run on a 1% agarose gel in TBE buffer (100mM Tris HCl, 90mM boric acid, 1mM EDTA) at a constant voltage of ~80V. The primers used to detect the *Gsh2*^{Cre} allele were TTGGCGCGCCTGTGAAGCGTTGGACAGAGGCCC and AGGTACAGGAGGTAGTCCCTC and gave a 600 base pair fragment in the presence of the transgene but no band in its absence. Primers used to detect the *Pax6*^{loxP} allele were AAATGGGGGTGAAGTGTGAG and TGCATGTTGCCTGAAAGAAG. The primers used to detect the RCE EGFP reporter allele were CCCAAAGTCGCTCTGAGTTGTTATC, GAAGGAGCGGGAGAAATGGATATG and CCAGGCGGGCCATTTACCGTAAG. PCR was performed using an MJ Research Tetrad thermal cycler.

2.4 Immunohistochemistry on paraffin embedded sections

Sections were dewaxed in xylene and rehydrated through an alcohol series, this included a 15 minute incubation step in 3% hydrogen peroxide/90% methanol in order to inactivate endogenous peroxidase activity. Sections were then washed in PBS and boiled for 20 minutes in 10mM sodium citrate (pH6) using a microwave to aid epitope recovery. Sections were washed in 0.1% Triton X-100/PBS (PBS-Tx) to permeabilise cells then blocked using 20% goat serum (Sigma) in PBS-Tx for 20 minutes. After blocking, sections were incubated with the primary antibody diluted in blocking solution overnight at 4^{o}C. Sections were then washed with PBS-Tx and incubated with a biotin conjugated goat anti-mouse secondary antibody diluted in blocking solution (1/200, Dako). Sections were washed in PBS-Tx then incubated in ABC avidin-biotin solution (Vector) for 1 hour at room temperature. After a further wash with PBS-Tx staining was performed using 0.05% diaminobenzidine (DAB) in tris buffered saline (Vector Laboratories). Following staining, sections were washed in water and dehydrated through an alcohol series before being mounted in DPX. Primary antibodies were used at the following concentrations; mouse anti-cleaved *Caspase-3* (1/50, Cell Signalling), mouse anti-*Islet1* (DSHB, Iowa City, Iowa, USA), mouse anti- *Lim1/2* (1/200 DSHB), mouse anti-*Mash1* (1/100), mouse anti-*Nkx2.2* (1/200 DSHB), mouse anti-*Pax6* (1/40 gift from V Van Heyningen, MRC Human Genetics Centre, Edinburgh, UK).

2.5 Fluorescent immunohistochemistry on paraffin embedded sections

Sections were dewaxed and boiled as above except that the hydrogen peroxide step was omitted. Following boiling sections were washed with PBS-Tx and blocked using 10% donkey serum (Sigma) in PBS-Tx for 20 minutes at room temperature. After blocking sections were incubated with the primary antibody/antibodies overnight at 4^{o}C. Sections were then washed with PBS-Tx and incubated with Alexa Fluor 488 donkey anti-goat secondary antibody (Invitrogen) and/or Alexa Fluor 568 donkey anti-mouse (Invitrogen) for 1 hour at room temperature, diluted 1/200 in blocking buffer. Sections were then washed with PBS-Tx and counterstained with

TOPRO-3 (Invitrogen) diluted 1/1000 in water. Sections were mounted in Vectashield Hardset (H-1400, Vector Laboratories). Primary antibodies used were goat anti-GFP (1/250 Abcam) mouse anti-*Pax6* (1/40).

2.6 Fluorescent immunohistochemistry on agarose embedded sections

Sections were washed with PBS-Tx to permeabilise cells and then blocked with 10% goat serum in PBS-Tx for 1 hour at room temperature. After blocking, sections were washed with PBS-Tx and incubated with the primary antibody/antibodies diluted in blocking buffer overnight at 4^{o}C. Sections were then washed for 3 x 30 minutes with PBS-Tx and incubated with Alexa Fluor 488 goat anti-rabbit secondary antibody (Invitrogen) and/or Alexa Fluor 568 goat anti-rat (Invitrogen) diluted 1/200 in blocking buffer overnight at 4^{o}C. Sections were washed for 3 x 30 minutes in PBS-Tx and counterstained with TOPRO-3 diluted 1/1000 in water before a final wash in PBS-Tx. Sections were mounted on Superfrost Plus slides in Vectashield (H-1000, Vector Laboratories) and sealed with nail polish. All steps were carried out in 24 well plates with shaking. Primary antibodies used were rabbit anti-GFP (1/1000 Abcam) and rat anti-L1 (1/500 Millipore).

2.7 In-situ hybridisation on paraffin sections

Tissue sections were dewaxed in xylene and transferred to a 50/50 xylene/ethanol solution before being rehydrated through an alcohol series. Sections were then washed in PBS and treated with proteinase K (20µg/ml) (Roche) for 15 minutes at 37^{o}C. Proteinase K activity was then terminated by incubation with 0.2% glycine and sections were washed with PBS. Postfixation was then performed using 4% PFA + 0.2% gluteraldehyde in PBS for 20 minutes at room temperature and washed with PBS. Sections then underwent prehybridisation by incubation in hybridisation mixture for 2 hours at 70^{o}C. Hybridisation mixture consisted of 50% formamide/5xSSC pH 7, 5mM EDTA, 10mg/ml blocking reagent (Roche), 1mg/ml baker's yeast tRNA (Roche) , 0.1 mg/ml heparin (BD Biosciences), 0.1% Tween20 and 0.1% Chaps (Sigma). Following prehybridisation sections were then hybridised

with digoxigenin-labelled riboprobes diluted in hybridisation mixture at 70°C overnight. After hybridisation sections were washed with 50% formamide/2xSSC at 70°C. Sections were further washed with PBS/0.1%Tween20 (PBST) before blocking for 1 hour at room temperature (1% blocking powder, 5% sheep serum in PBST). Sections were then incubated with anti-DIG AP fragments antibody (Roche) diluted 1/1000 in blocking buffer for 2 hours at 37°C. Following the antibody step sections were washed with NTM (100mM NaCl, 100mM Tris HCl, pH9.5, 50mM $MgCl_2$). Staining was then carried out using NBT/BCIP solution (Roche) diluted 1/50 in NTM for between 6 and 24 hours. After staining was deemed to be complete sections were washed in PBS and mounted in Aquatex mounting medium (VWR). Riboprobes used were complimentary to *Dbx1* (gift from Thomas Theil), *Ngn2* or *Sonic Hedgehog*.

2.8 In-situ hybridisation on agarose embedded sections

After collection in PBS, sections were mounted on Superfrost plus slides and allowed to dry at room temperature. Sections were then incubated in methanol to remove agarose and rehydrated through an alcohol series. Sections were washed with 1%Tween20 in tris-buffered saline (TBST) and bleached with 6% hydrogen peroxide in TBST. Sections were then treated with with proteinase K (5µg/ml) for 15 minutes at room temperature. Proteinase K activity was then terminated by incubation with 0.2% glycine for 20 minutes and sections were washed with TBST. Postfixation was then performed using 4% PFA in TBS for 30 minutes. Sections then underwent prehybridisation by incubation in hybridisation mixture for 2 hours at 65°C. Hybridisation mixture consisted of 50% formamide / 5x SSC, 50µg/ml baker's yeast tRNA, 50µg/ml Heparin and 1% SDS. After prehybridisation sections were then hybridised with digoxigenin-labelled riboprobes diluted in hybridisation mixture at 65°C overnight. Following hybridisation the sections were washed in 50% formamide/5xSSC 3 x 20minutes at 65°C. Sections were then washed with TBST and blocked with 10% sheep serum in TBST for 1 hour at room temperature. Sections were incubated with anti-DIG AP fragments antibody diluted 1/1000 in blocking buffer overnight at 4°C. After the antibody step sections were washed with TBST 5 x 90 minutes then washed with NTM. Staining was then carried out using

NBT/BCIP solution diluted 1/50 in NTM for between 6 and 36 hours. When staining was deemed to be complete sections were washed with PBS and mounted in 90% glycerol in PBS. Riboprobes used were complimentary to *Slit1, Slit2, Robo1 and Robo2*.

2.9 Quantitative real time PCR

Tissue samples from the thalamus and hypothalamus of WT and *Pax6*$^{Sey/Sey}$ embryos were collected and flash frozen on dry ice. Total RNA was then extracted from the tissue samples using an RNAeasy Mini kit (Qiagen). The total RNA was then used to create cDNA using Superscript reverse transcriptase (Invitrogen). Using a Qiagen Quantitect SYBR Green PCR kit (Qiagen), quantitative real time PCR (qRT-PCR) analysis was then carried out with the following primer pairs: *Slit1* (5'-CCTGCCAGATGATCAAGTGC-3' and 5'-GCTGCTTCTGGTAATAGTCC-3'), *Slit2* (5'-TCACTGACCTGCAGAACTGG-3' and 5'-ACCATCTGGTCGAAGGTGAC-3'), *Robo1* (5'-GCCACTTCCATGCCTCTCAG-3' and 5'-GTGCCTTGGACTGGACAGTG-3'), *Robo2* (5'-GCAGAAGTAAACCGGACGAA-3' and 5'-CTCCAAGATTGCAGGCTCTC-3'). The PCR reaction was carried out using an MJ Research Opticon Light Cycler. The abundance of each transcript (relative to *GAPDH*) was calculated using Opticon software and Microsoft Excel.

2.10 DiI and DiA injection

Whole brains were dissected at E14.5, E16.5 or E18.5 and fixed for at least 2 days with 4% PFA in PBS at 4^{o}C, with shaking. After fixation brains were washed with PBS. For thalamic injection the brains were cut in half at the midline in the sagittal plain and a small hole was made in the medial aspect of the thalamus using a fine probe. A small (<200µm^2) peice of DiI impregnated filter paper (Neurovue Red, Molecular Targetting Technologies Inc) was cut using a fine blade and inserted into the prepared hole in the thalamus. For cortical or ventral telencephalic injection, holes were made in the desired region of the telencephalon without any further

dissection of the brain and small peices of either DiI or DiA impregnated filter paper (Neurovue Jade, Molecular Targetting Technologies Inc) were inserted. The whole or half brains were then incubated in PBS at 37^{o}C to allow the dye to diffuse. E14.5 brains were incubated for 1 week while E16.5 and E18.5 brains were incubated for 3-4 weeks. After diffusion the brains were embedded in agarose and sectioned either coronally (telencephalic injections) or at a 45-60^{o} angle (thalamic injections) at 100µm using a vibratome. Sections were counterstained with TOPRO-3 diluted 1/1000 in PBS for 30 minutes with shaking, and then washed with PBS. Sections were then mounted on Superfrost Plus slides in Vectashield (H1000); slides were sealed using nail polish.

2.11 Imaging

Bright field images of DAB stained sections or *in-situ* hybridisation were captured using a Leica DLMB microscope and a DFC480 colour digital camera. Leica imaging software was used during image acquisition. Fluorescent images were captured using either a Leica AF6000 microscope connected to a DFC360 FX digital camera with Leica Advanced Fluorescence imaging software or a Leica NTS confocal microscope with Leica Confocal Software. The brightness and contrast of some images was modified using Adobe Photoshop.

2.12 Quantification of cell number from DAB stained sections

The number of DAB stained cells present in the prethalamus sections after immunohistochemistry for *Pax6* and cleaved-*Caspase3* was performed in the following manner. Non-adjacent sections at 60µm intervals were imaged for counting; only sections in which the diencephalon was connected to the ventral telencephalon were used for counting (4 sections per embryo). DAB stained cells were then counted throughout the region in which the *Gsh2*^{Cre} was active on one side of the midline of the brain. To define this region images from adjacent sections on which GFP immunohistochemistry was performed were overlayed. The mean number of stained cells counted per section was then calculated for the embryo. This

method was used to quantify *Pax6* positive cells present within the prethalamus chapter 5, figure 1 and Cleaved-C*aspase3* positive cells within the prethalamus in chapter 5, figure 11.

2.13 Quantification of prethalamic pioneer axon outgrowth

Pioneer axon outgrowth from the prethalamus was quantified using images from sections on which GFP immunohistochemistry had been performed. Images were captured while maintaining a constant exposure time and gain. Three non-adjacent sections were quantified for each embryo, and three control embryos and three $Pax6^{cKO}$ embryos were quantified at each developmental stage analysed.

Using *ImageJ* image analysis software a line approximately 200µm in length was placed on the tissue in a region not deemed to contain axons (Fig. 1A). Using the 'plot' function a plot of the fluorescence intensity along the line was then drawn. The highest intensity value along this line (red circle, Fig. 1B) was then used as the background fluorescence value. To quantify prethalamic axon outgrowth a horizontal line was drawn crossing the thalamus 100µm dorsal to the prethalamus (Fig. 1C). The intensity of the fluorescence was then plotted along the line (Fig. 1D), peaks correspond to axons crossing the line. The line is deemed to be covered by an axon where it is above the background value (red line, Fig. 1D). The percentage of the line covered by axons was calculated using *Microsoft Excel*. This method of quantification is a modified version of that used by Tian et al (2008) and Chen et al (2012) to quantify axon outgrowth from cultured explants. Note, the n number given in the text refers to the number of animals used not the number of sections or images quantified.

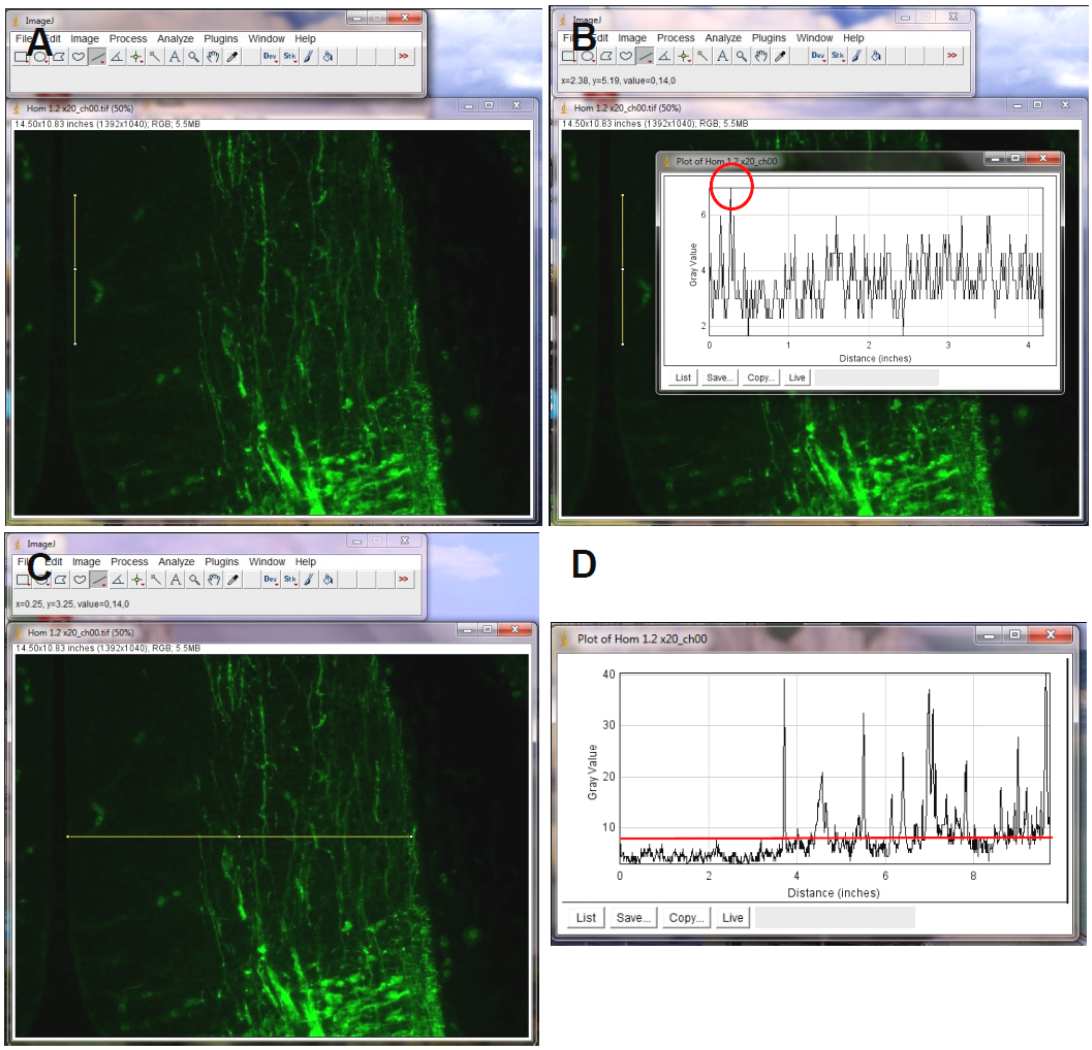


***Figure 1*. Method used for the quantification of prethalamic axon outgrowth.**

Chapter 3: The development of the thalamocortical tract in *Pax6*$^{Sey/Sey}$ embryos

3.1 Introduction

The cortex receives most of its sensory input from the periphery via the thalamus. It is the thalamocortical tract that is responsible for relaying this sensory information from the thalamus to the cortex.

The formation of this axon tract is achieved by the guidance of growing axons from their cell bodies within the thalamus to their target region in the cortex. The route taken by these axons is a complex one in three dimensions. Initially axons extend ventrally through the prethalamus; they then turn into the ventral telencephalon crossing the diencephalic-telencephalic boundary (DTB), and finally turn once again to reach the cortex crossing the pallial-subpallial boundary (PSPB). At the same time axons extend from the cortex along the same path to form reciprocal connections with the thalamus (Lopez-Bendito and Molnar, 2003). The complexity of the route taken by the extending thalamocortical axons (TCAs) makes the thalamocortical tract an excellent model by which to study the processes of axon guidance.

There are several methods by which TCAs are guided to their targets within the cortex. The expression of a number of chemoattractive or chemorepellent guidance cues and their receptors at specific points along the route of the thalamocortical tract has been show to be particularly important for guiding TCAs. An example of this is the expression of repulsive cues, *Slits*, at the hypothalamus and their receptors on the growth cone, the *Robos*. Signalling between the *Slit* ligand and the *Robo* receptor causes the deflection of axons away from the hypothalamus and towards the telencephalon (Bagri et al., 2002; Braisted et al., 2009; Lopez-Bendito et al., 2007). TCAs are also thought to be guided along so-called ‘pioneer axons’ that extend from populations of cells in the ventral telencephalon and the prethalamic reticular nucleus. TCAs are proposed to use these axons as a ‘scaffold’ in order to reach the telencephalon (Braisted et al., 1999; Molnar et al., 1998a).

A variety of transcription factors have been shown to have an important role in the development of the thalamocortical tract. Knockout studies have shown that a number of different transcription factors are required for the correct formation of the thalamocortical tract. These include *Emx2*, *Dlx1/2*, *Mash1* and *Pax6* (Garel et al.,

2002; Lopez-Bendito et al., 2002; Pratt et al., 2000b; Tuttle et al., 1999). In many of these knockout mice the thalamocortical tract is severely malformed.

Several studies have been conducted examining the effect on the thalamocortical tract of the constitutive loss of *Pax6*. A number of different models have been used including the small eye (*Pax6*$^{Sey/Sey}$) mouse (Hogan et al., 1988), the small eye rat (Matsuo et al., 1993) and the *Pax6/LacZ* knockout mouse (St-Onge et al., 1997). Axon tracing experiments using these models have shown that the development of the thalamocortical tract is severely affected by the loss of *Pax6*. TCAs do not reach the cortex and some axons head towards the hypothalamus. In addition some of these studies have reported that some TCAs reach the ventral telencephalon but do not cross the PSPB (Hevner et al., 2002; Jones et al., 2002; Kawano et al., 1999; Pratt et al., 2002)

Co-culture experiments performed by Pratt et al (2000) have shown that TCAs that lack *Pax6* are able to grow into WT ventral telencephalon but they do not extend as far as WT TCAs and fail to turn toward the cortex. This demonstrates that *Pax6* is required in the thalamus and suggests that axon guidance defects seen in the *Pax6*$^{Sey/Sey}$ embryo are a result of an autonomous change in cells with cortical projections. It is also possible that *Pax6* is influencing the guidance of TCAs in a non autonomous manner by affecting the environment through which TCAs grow. Evidence for this comes from the fact that in the cortex of *Pax6*$^{LacZ/KO}$ mice the expression of attractive guidance cues *Sema3C* and *Sema5A* are reduced (Jones et al., 2002).

In this chapter the *Pax6*$^{Sey/Sey}$ mouse has been used as a model to further understand the role that *Pax6* plays in the development of the thalamocortical tract. Firstly a combination of axon tract tracing and immunohistochemistry experiments were performed to determine the exact nature of the TCA path-finding defects seen in embryos that lack *Pax6*. Secondly the development of the TCA permissive 'corridor' was examined in *Pax6*$^{Sey/Sey}$ embryos. Thirdly *In-situ* hybridisation and quantitative real time PCR were used to examine the expression of *Slit* and *Robo* guidance cues in the *Pax6*$^{Sey/Sey}$ embryo.

3.2 Results

3.2.1 Thalamocortical axon path-finding defects in *Pax6*$^{Sey/Sey}$ mice

In order to examine the thalamocortical tract of WT and *Pax6*$^{Sey/Sey}$ mice, carbocyanine dye DiI was injected into the thalamus of E14.5 and E18.5 brains. In the WT condition at E14.5 TCAs can be seen leaving the thalamus, extending in a ventral direction through the prethalamus before turning laterally and crossing the DTB to reach the ventral telencephalon (Fig. 1A). By E18.5 the TCAs have extended through the ventral telencephalon and made a second turn dorsally, crossing the PSPB to reach the cortex (Fig. 1C).

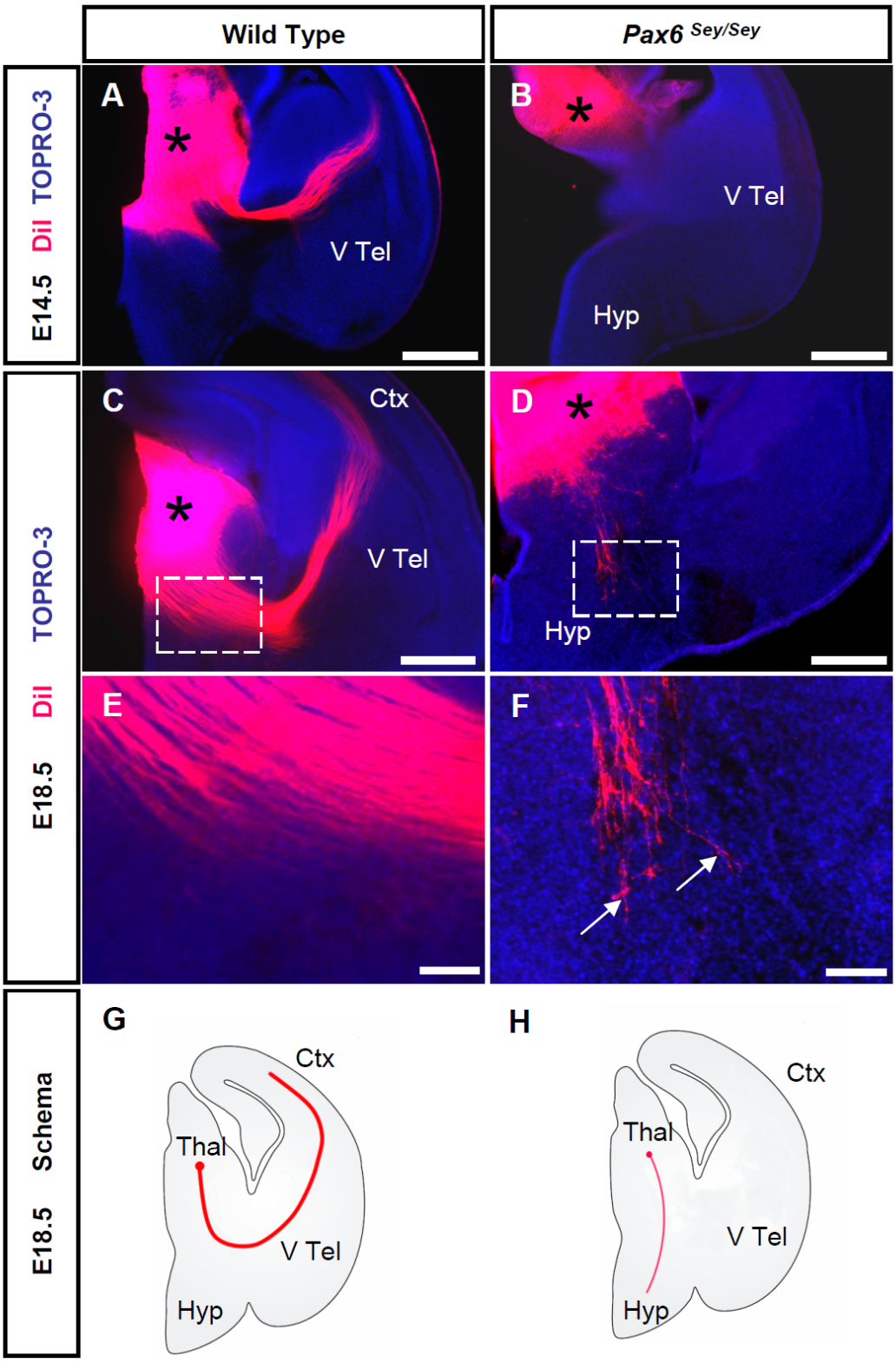
In *Pax6*$^{Sey/Sey}$ mice at E14.5, unlike in the WT, no DiI labelled axons can be seen within the ventral telencephalon, in fact at this stage no TCAs have been observed leaving the thalamus (n = 4) (Fig. 1B). At E18.5 DiI diffusion reveals a small number of growth cone tipped TCAs leaving the thalamus and extending ventrally through the prethalamus (arrows Fig.1F), but unlike in the WT these axons do not make a lateral turn towards the ventral telencephalon but instead head towards the hypothalamus (Fig. 1D, F), a region normally repulsive to thalamocortical axons (Lopez-Bendito et al., 2007; Pratt et al., 2000b). No DiI labelled axons are observed in the ventral telencephalon (n = 4) (Fig. 1D).

This result is broadly in line with the findings of other studies looking at the thalamocortical tract of *Pax6* mutant mice (Pratt et al, 2002; Hevner at al, 2002; Jones et al 2002) but while other studies report that some TCAs cross the DTB and reach the ventral telencephalon, this axon tracing experiment provides no evidence that this is the case.

Table 1. A breakdown of the number of animals used in each of the experiments conducted as part of this chapter.

			n number	
Figure	Experiment	Age	WT	*Pax6*$^{Sey/Sey}$
1	Thalamic DiI placement	E14.5 E18.5	3 3	4 4
2	Cortical DiI placement	E18.5	2	3
3	*L1* Immunohistochemistry	E14.5 E16.5 E18.5	3 3 3	5 3 3
4	Ventral telencephalon DiI placement	E16.5	3	4
5	PSPB/Lateral telencephalon DiI placement	E16.5	2	4
6	*Islet1* immunohistochemistry	E14.5	2	3
	Nkx2.1 immunohistochemistry	E14.5	2	3
7	*Slit1 in situ* hybridisation	E14.5	3	3
	Slit1 qRT-PCR	E14.5	5	5
	Slit2 in situ hybridisation	E14.5	3	3
	Slit2 qRT-PCR	E14.5	5	5
8	*Robo1 in situ* hybridisation	E14.5	3	3
	Robo1 qRT-PCR	E14.5	5	5
	Robo2 in situ hybridisation	E14.5	3	4
	Robo2 qRT-PCR	E14.5	5	5

Figure 1. Thalamocortical axon path finding defects in *Pax6*$^{Sey/Sey}$ mice. (A,C) DiI placement in the thalamus of WT mice reveals thalamocortical axons extending into the ventral telencephalon reaching the PSPB at E14.5 (A) and entering the cortex by E16.5 (C). **(B,D)** DiI placement in the thalamus of *Pax6*$^{Sey/Sey}$ mice shows no axons leaving the thalamus at E14.5 (B) at E16.5 a small number of thalamocortical axons can be seen heading towards the hypothalamus, while no axons are observed within the ventral telencephalon (D). **(E, F)** Higher magnification images of boxed regions in C and D respectively. **(G,H)** Schematic diagram illustrating the thalamocortical axon path-finding defects observed in the *Pax6*$^{Sey/Sey}$ mouse. Asterisk denotes DiI injection site. Scale bars: 500μm in A-D, 100μm in E and F.


Wild Type
Pax6 Sey/Sey
E14.5 DiI TOPRO-3
E18.5 DiI TOPRO-3
E18.5 Schema
A
B
C
D
E
F
G
H
V Tel
Hyp
Ctx
Thal

3.2.2 Corticothalamic axon pathfinding defects in the *Pax6*$^{Sey/Sey}$ mice

After investigating the behaviour of thalamocortical axons in *Pax6*$^{Sey/Sey}$ mice the corticothalamic axons were examined in a similar fashion using carbocyanine dye. DiI was placed within the cortex of WT and *Pax6*$^{Sey/Sey}$ mice at age E18.5. In WT mice this labels both corticothalamic and thalamocortical axons by anterograde and retrograde DiI diffusion respectively (Fig. 2A). As well as TCAs, the cell bodies of these axons are also labelled within the thalamus (Fig. 2D).

When DiI was placed in the cortex of *Pax6*$^{Sey/Sey}$ mice a small number of corticothalamic axons are labelled. These axons can be seen crossing the presumptive PSPB and entering the ventral telencephalon (Fig. 2B, arrow 2E). These axons continue in a ventral direction towards the ventral surface of the brain (arrow Fig. 2F). Unlike the WT no TCAs or cell bodies within the thalamus have been labelled by this experiment as no TCAs reach the cortex in the *Pax6*$^{Sey/Sey}$ mouse (Fig. 1) (Jones et al, 2002; Pratt et al, 2002; Hevner et al, 2002).

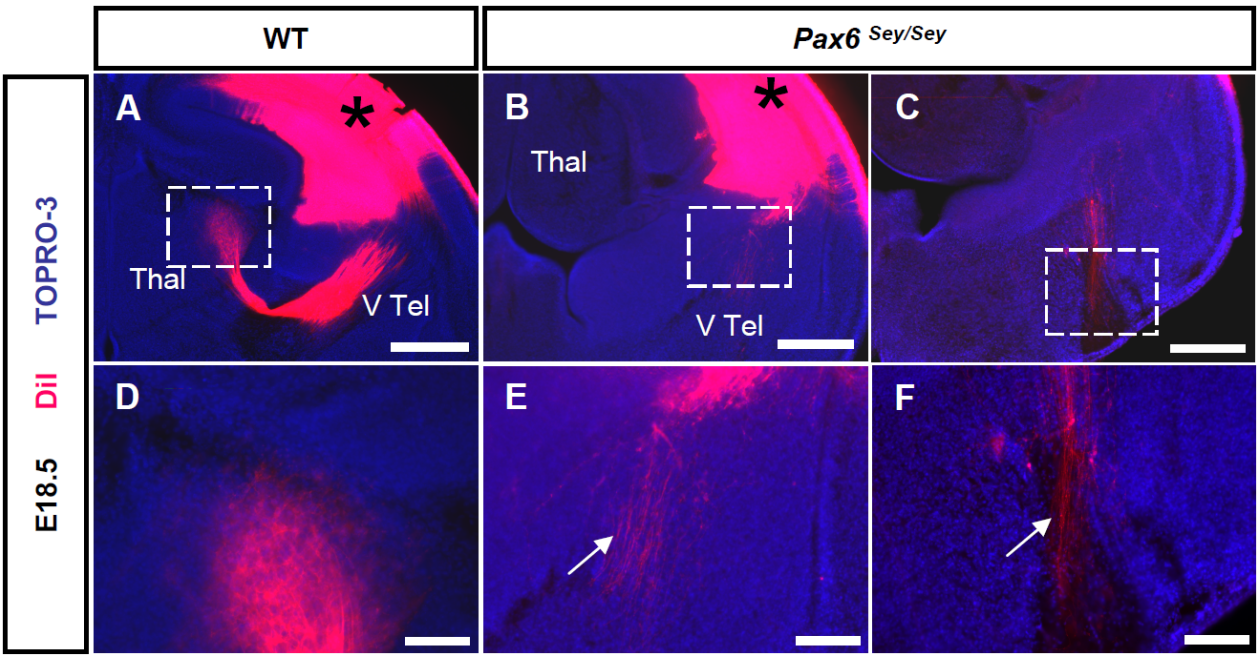


Figure 2. Corticothalamic axon path finding defects in *Pax6*$^{Sey/Sey}$ mice. (A,D) DiI placement in the WT cortex at E18.5 labels the thalamocortical tract and corticothalamic axons (A). Cell bodies within the thalamus are also labelled by retrograde DiI diffusion (D). **(B,C,E,F)** DiI placement in the *Pax6*$^{Sey/Sey}$ cortex labels a small number of corticothalamic axons leaving the cortex (B,E). At a further rostral level cortical axons can be seen heading towards the ventral surface of the ventral telencephalon (C,F). D,E and F are higher magnification images of boxed areas in A,B and C respectively. Asterisk denotes DiI injection site. Scale bars: 500μm in A-C, 100μm in D-F.

3.2.3 L1 immunohistochemistry

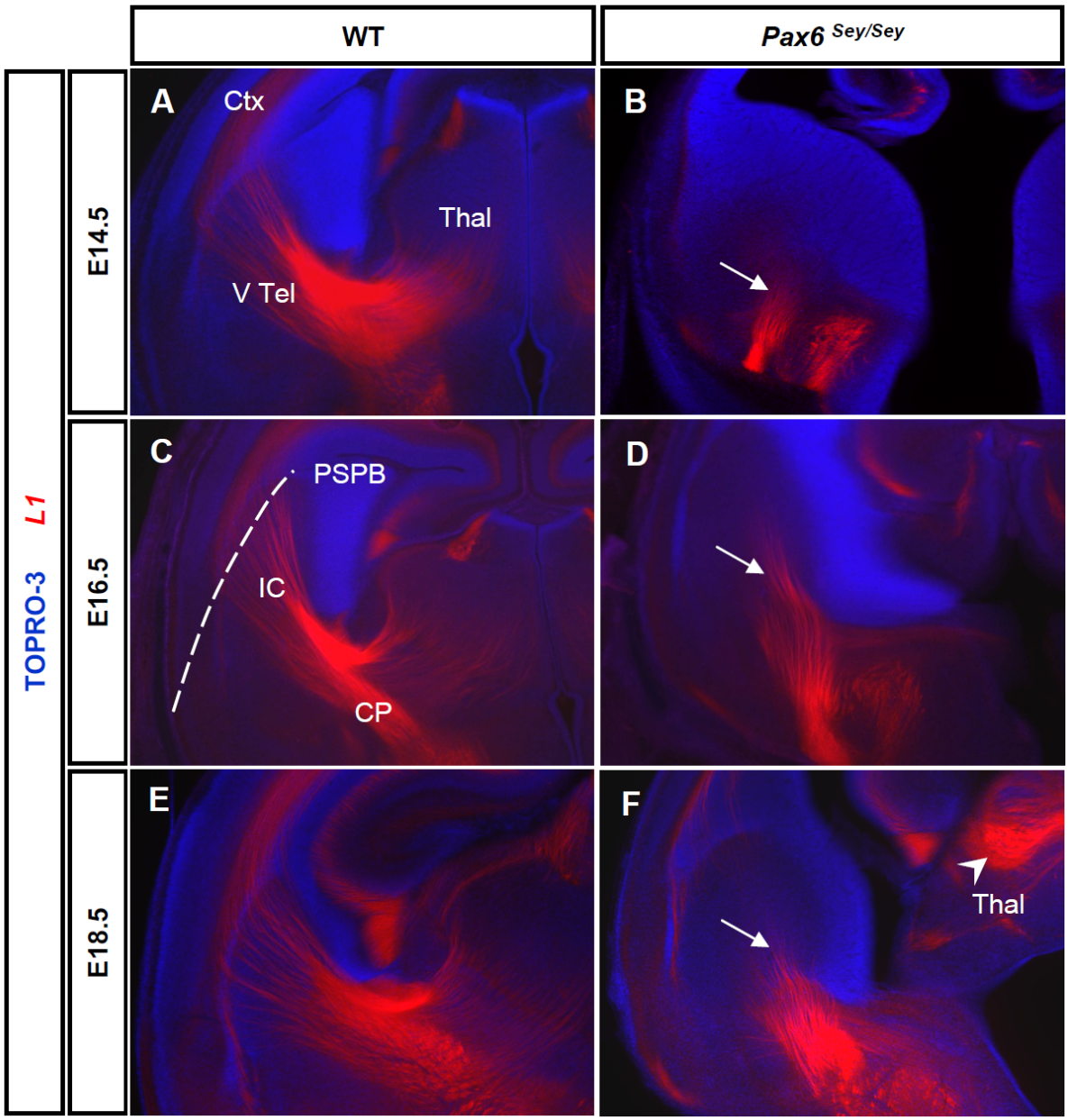
In order to further investigate the thalamocortical tract in the *Pax6*$^{Sey/Sey}$ mouse immunohistochemistry for axonal marker L1 was performed at E14.5, E16.5 and E18.5. *L1* is a cell adhesion molecule and a marker of major axonal tracts such as the thalamocortical tract and the corpus callosum. From E14.5 *L1* is expressed by both TCAs and corticothalamic axons (CTAs). This technique is useful in that it allows for the visualisation of the entire axon tract, but it is limited by the fact that it is not possible to distinguish between the various different axonal populations such as TCAs, CTAs and corticospinal axons.

In the WT at E14.5 the thalamocortical tract can be clearly identified as the large fibre bundle leaving the thalamus and turning into the ventral telencephalon. By this point the TCAs have reached, but not yet crossed, the PSPB. When the tract crosses the DTB it forms a tight fascicle, but as the axons approach the PSPB they defasciculate to form the fan like structure of the internal capsule. *L1* staining within the cortex labels CTAs within the intermediate zone extending towards the PSPB (Fig. 3A). By E16.5 the TCAs have crossed the PSPB to reach the cortex. At this stage CTAs have crossed the PSPB and have mingled with the TCAs within the internal capsule, some CTAs will have reached the diencephalon but they will not yet have reached their targets in the thalamus. At E16.5 corticospinal axons can also be observed heading through the cerebral peduncle *en route* to the spinal cord (Fig. 3C). By E18.5 both TCAs and CTAs have reached their target regions in the cortex and thalamus respectively (Fig. 3E). In contrast the pattern of *L1* staining is entirely different in the *Pax6*$^{Sey/Sey}$ mouse brain. At E14.5 a small *L1* positive bundle of axons is present within the ventral part of the ventral telencephalon adjacent to the amygdaloid region (Fig. 3B). At E16.5 this bundle of axons has become larger and extends from the ventral edge of the ventral telencephalon towards the PSPB (Fig. 3D). This large bundle is still present at E18.5. At this stage a large mass of TCAs can be seen within the thalamus which may explain why so few TCAs have been observed leaving the thalamus (Fig. 3F).

This axon bundle observed within the *Pax6*$^{Sey/Sey}$ ventral telencephalon has been reported by other studies to be TCAs (Jones et al, 2002; Kawano et al, 1999). However the result from the DiI injection experiment (Fig.1) does not support this as

no DiI labelled axons have been found in the ventral telencephalon when DiI is injected into the thalamus.

Figure 3. Immunohistochemistry for axonal marker *L1* reveals a large axon tract present within the ventral telencephalon of *Pax6*$^{Sey/Sey}$ mice. (A,C,E) Immunohistochemistry for *L1* shows the thalamocortical tract of WT mice at E14.5 (A) E16.5 (C) and E18.5 (E). **(B,D,F)** *L1* staining of *Pax6*$^{Sey/Sey}$ brains reveals a large axon tract within the ventral telencephalon (arrows B, D, F). This tract is first observed at E14.5 (B) and is larger at E16.5 and E18.5 (D, F). Scale bar: 500μm.


WT
Pax6 Sey/Sey
TOPRO-3 L1
E14.5
E16.5
E18.5
A
Ctx
Thal
V Tel
B
C
PSPB
IC
CP
D
E
F
Thal

3.2.4 A large aberrant axonal tract within the ventral telencephalon of *Pax6*$^{Sey/Sey}$ brains is not made up of thalamic axons.

In order to determine the origin of the L1 positive axon tract found within the ventral telencephalon DiI was placed at two distinct points, firstly at the ventral aspect of the ventral telencephalon and secondly close to the PSPB. In this way it is possible to label both the cell bodies and tips of the growing axons by anterograde and retrograde DiI diffusion.

In an attempt to label this tract DiI was placed in the ventral telencephalon medial to the amygdaloid region. This is where the axon tract appears to originate from according to the L1 immunohistochemistry (Fig. 3). In WT mice DiI placement in this region does not label any major axon tract within the ventral telencephalon. DiI labelling can be seen surrounding the site of injection (asterisk Fig. 4A) but no distant axons or cell bodies are labelled (Fig 4A, G).

DiI placement at this site in *Pax6*$^{Sey/Sey}$ mice labels the large axon tract that has been observed using L1 immunohistochemistry. Labelling can be seen across a large area within the ventral telencephalon, extending dorsally towards the PSPB (Fig. 4B). Looking in the sagittal plane it can be seen that the tract extends rostrally as well as dorsally (arrow Fig. 4H). Close to the PSPB a large number of cell bodies can be seen (arrows Fig. 4E). This is in contrast to the WT where no DiI labelling can be observed at this position (Fig. 4D). These cells have presumably been labelled by retrograde DiI diffusion; this indicates that at least some of the axons within this aberrant tract originate from these cells close to the PSPB.

Despite observing a large number of DiI labelled cell bodies close to the PSPB no labelled cell bodies or axons have been observed in the thalamus (n = 4) (Fig. 4F). This finding shows that the axons seen in the ventral telencephalon are not in fact TCAs but rather they originate from cells within the ventral telencephalon itself.

When DiI was placed close to the PSPB at E16.5 in WT mice the thalamocortical tract was labelled as might be expected at this stage (Fig. 5A). The axons of the internal capsule can be clearly seen and cell bodies within the thalamus are also labelled (arrow Fig. 5C). In *Pax6*$^{Sey/Sey}$ embryos DiI placement in this region labels the axon tract seen in the previous experiments (Fig. 3, 4). The axon bundle

can clearly be seen extending towards the ventral surface of the brain (Fig. 5B). At the most ventral extent of the axon bundle growth cone tipped axons can be seen (arrows Fig. 5D) indicating that this is the growing 'front' of the axonal tract.

Taken together, the data thus far in this chapter show that in mice that lack functional *Pax6* few TCAs leave the thalamus and the small number of axons that do head towards the hypothalamus. Furthermore an axon bundle within the ventral telencephalon that has previously been assumed to consist of TCAs (Jones et al, 2002; Hevner et al, 2002) is in fact made up of axons that originate from cells found within the ventral telencephalon itself.

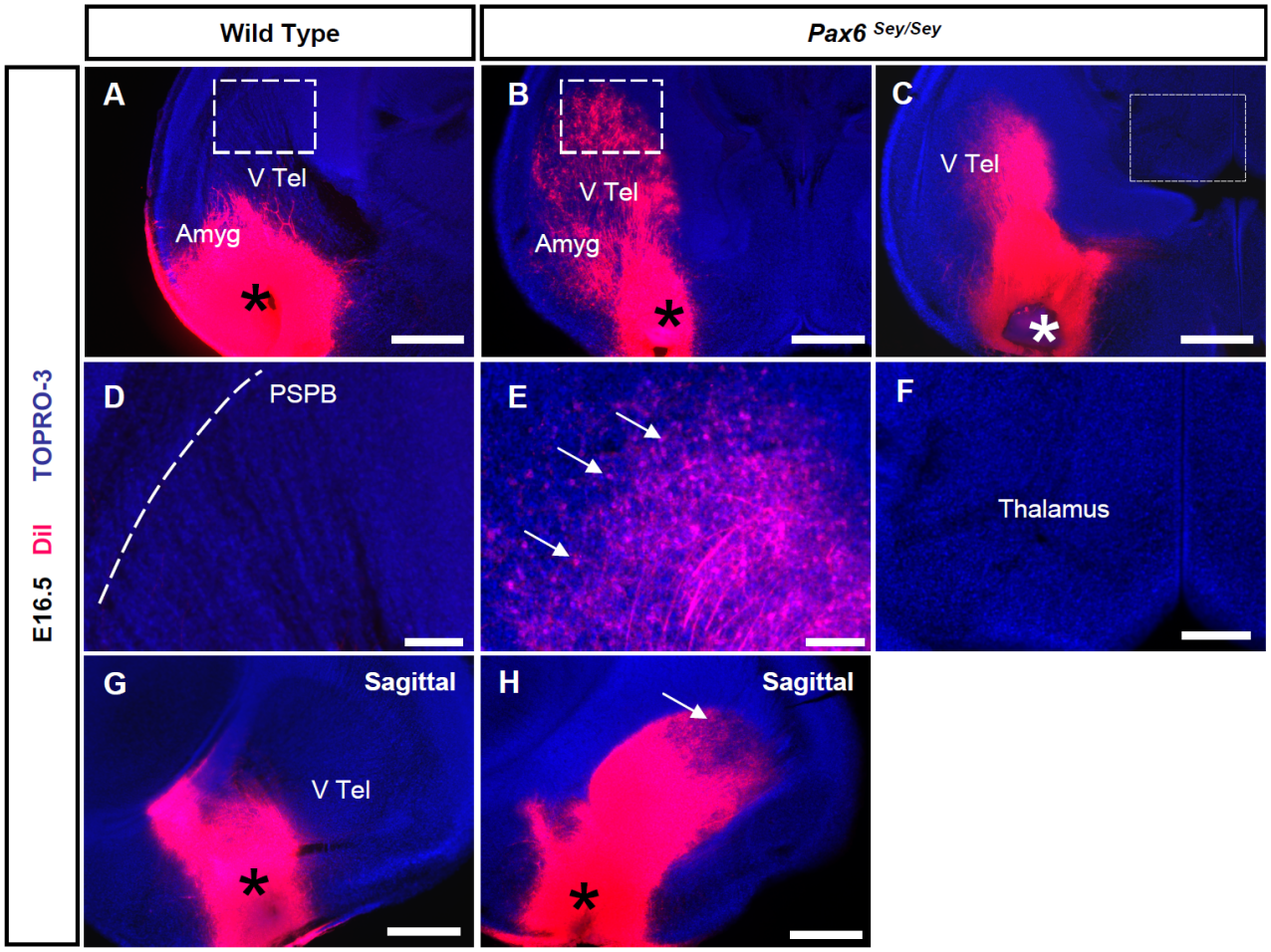


Figure 4. DiI Injection adjacent to the amygdaloid region labels a large abberant tract within the ventral telencephalon in the *Pax6*$^{Sey/Sey}$ mouse. (A, D, G) DiI placement close to the amygdaloid region in WT mice does not label any major axon tract. **(B, E, H)** DiI placement in *Pax6*$^{Sey/Sey}$ mice labels a large axon tract within the ventral telencephalon (B) and a large number of cell bodies close to the PSPB (arrows, E), this tract extends in a rostral direction from the injection site (H). **(C, F)** DiI placement does not label any axons or cell bodies within the thalamus of *Pax6*$^{Sey/Sey}$ mice. D,E and F are higher magnification images of boxed regions in A,B and C respectively. Asterisk denotes DiI injection site. Scale bars: A-C, G, H 500µm D-F 100µm.

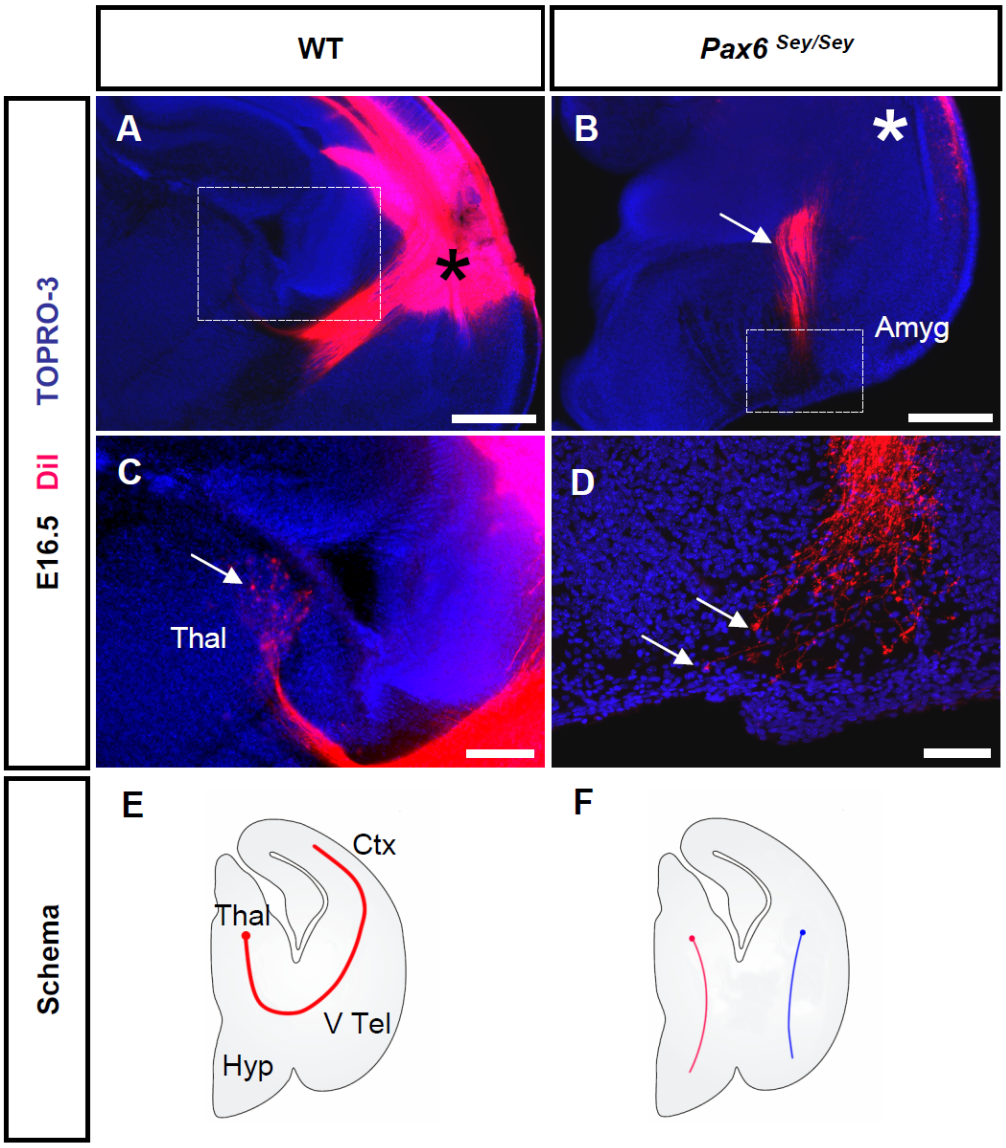


Figure 5. **DiI Injection close to the PSPB in the *Pax6*$^{Sey/Sey}$ mouse labels a large axon tract that extends ventrally. (A,C)** DiI injection close to the PSPB in WT mice labels the thalamocortical tract (A) and cell bodies within the thalamus (C) due to retrograde DiI diffusion. **(B,D)** DiI injection in *Pax6*$^{Sey/Sey}$ mice reveals a large bundle of axons that extend ventrally (B), these axons are tipped with growth cones (D). **(E,F)** Schematic diagram illustrating the route of the thalamocortical tract (red) and the aberrant tract found within the *Pax6*$^{Sey/Sey}$ mouse (blue). C and D are higher magnification images of the boxed regions in A and B respectively Asterisk denotes DiI injection site. Scale bars: A and B 500µm C and D 100µm.

3.2.5 The axon-permissive 'corridor' within the ventral telencephalon is disrupted in *Pax6*$^{Sey/Sey}$ embryos

The tract tracing experiments above have demonstrated that TCAs fail to enter the ventral telencephalon in *Pax6*$^{Sey/Sey}$ embryos. A possible explanation for this is that the axon guidance cues present within the ventral telencephalon are altered in *Pax6*$^{Sey/Sey}$ embryos thereby preventing TCAs from crossing the DTB to reach the telencephalon. An important mechanism by which TCAs are guided through the ventral telencephalon is the formation of the so-called 'corridor' cells which migrate from the LGE to the MGE and express the cell membrane bound form of guidance cue *Neuregulin1*. This provides a permissive territory for TCA growth through the otherwise non-permissive MGE, the repulsive character of the MGE is possibly due to expression of *Slit1*. In mutant embryos where the corridor does not form (such as those deficient in *Mash1*), TCA entry to the telencephalon is disrupted (Bielle et al., 2011a; Lopez-Bendito et al., 2006). Simpson et al (2009) showed that in embryos where *Pax6* expression was specifically reduced in ventral telencephalon the corridor becomes abnormally widened, and that a number of TCAs are unable to cross the DTB.

To determine if the formation of the corridor is disrupted in *Pax6*$^{Sey/Sey}$ embryos immunohistochemistry was performed at E14.5 for *Islet1* to mark the corridor cells and *Nkx2.1* to mark the MGE and globus pallidus (GP). In WT embryos the *Islet1* expressing corridor can clearly be seen extending from the LGE towards the diencephalon (Fig. 6A) while *Nkx2.1* expression can be seen above the corridor in cells of the MGE and below at the GP (Fig. 6B). In *Pax6*$^{Sey/Sey}$ embryos the *Islet1* positive corridor appears much less well defined than in the WT and extends abnormally ventrally (arrows, Fig. 6C). The *Nkx2.1* expression within the MGE covers a much larger area of the ventral telencephalon much larger and also ventrally along the medial edge of the ventral telencephalon (arrows, Fig. 6D). This confirms that the formation of the corridor is indeed malformed in the absence of *Pax6*$^{Sey/Sey}$ embryos. This, combined with the expansion of the repulsive MGE, may act to prevent TCA entry into the telencephalon. This data also provides further evidence that *Pax6* plays a crucial role in the formation of the axon permissive corridor.

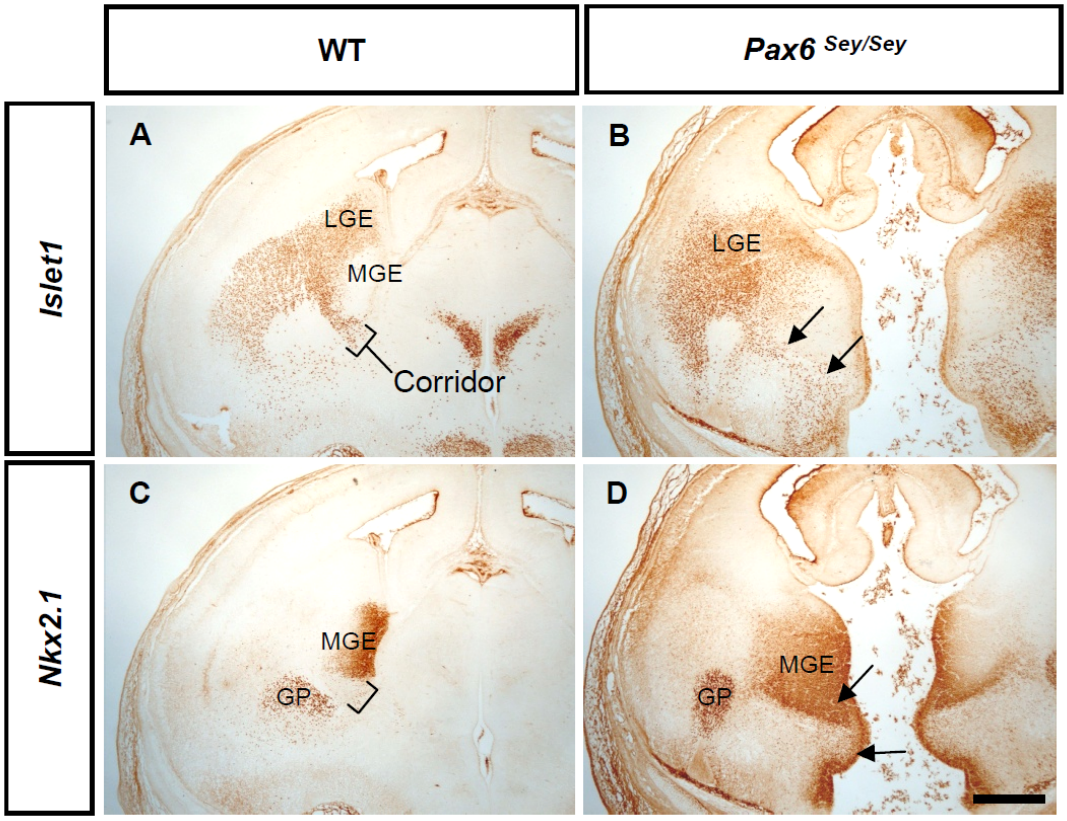


Figure 6. **Development of the TCA-permissive 'corridor' is disrupted in the ventral telencephalon of *Pax6*$^{Sey/Sey}$ embryos**. **(A,B)** At E14.5 immunohistochemistry for *Islet1* marks cells of the LGE and cells which migrate to form the corridor within the MGE. In WT embryos the corridor can be clearly seen extending to the diencephalon (A), in *Pax6*$^{Sey/Sey}$ embryos the corridor is less well defined and extends in an abnormally ventral direction (arrows, B). **(C,D)** *Nkx2.1* expression marks cells of the MGE and globus pallidus (GP), which are repulsive to TCAs. In WT embryos *Nkx2.1* expression can be seen above and below the corridor (C), in *Pax6*$^{Sey/Sey}$ embryos *Nkx2.1* expression is expanded laterally and dorsally from the MGE (arrows, D). C and D are adjacent sections to A and B respectively. Scale bar: 500µm.

3.2.6 Expression of *Robo2* is reduced in the *Pax6*$^{Sey/Sey}$ thalamus while *Slit* expression is maintained at the hypothalamus

The expression of a variety of axon guidance molecules and their receptors is required for the correct formation of the thalamocortical tract. There are several families of genes that are involved in axon guidance. These include the *Slits, Robos, Semaphorins* and *Plexins* (Bagri et al., 2002; Dufour et al., 2003; Little et al., 2009; Suto et al., 2005). The axon tract tracing experiments showed that in the *Pax6*$^{Sey/Sey}$ brain thalamic axons do not reach the ventral telencephalon but instead invade the hypothalamus. One possible explanation for this is that the expression of key guidance molecules is disrupted in the *Pax6*$^{Sey/Sey}$ brain causing the axon guidance defects previously observed. There is already evidence to suggest that the expression of guidance cues may be altered in mice that lack *Pax6*. Jones et al (2002) describe how the expression patterns of members of the semaphorin family *Sema5A* and *Sema3C,* are altered in the *Pax6*^{LacZKO} telencephalon.

In *Pax6*$^{Sey/Sey}$ mice TCAs enter the hypothalamus (Fig. 1); this is a region that is normally repulsive to TCAs (Pratt et al, 2000; Bagri et al, 2002). This repulsive character is due to signalling between the *Slits* and their receptors on the growth cone the *Robos* (Lopez-Bendito et al, 2007; Braisted et al, 2009). It is possible that the expression of the *Slit* and *Robo* genes is altered in the *Pax6*$^{Sey/Sey}$ mouse. To test this hypothesis, in-situ hybridisation was used to detect any change in the expression pattern of these guidance cues and quantitative real time PCR (RT-PCR) was carried out to assess gene expression levels.

Slit 1 and *2* are diffusible axon guidance cues that have been shown to have a repulsive effect on a variety of growing axons including retinal ganglion cell axons, commissural spinal axons and TCAs (Braisted et al., 2009; Brose et al., 1999; Erskine et al., 2000; Plump et al., 2002). In the WT mouse brain at E14.5, *Slit1* mRNA is expressed primarily at the midline of the diencephalon, in the prethalamus and in the hypothalamus. Expression is also seen in the developing cortical plate and at the ventricular zone of the ventral telencephalon (Fig. 7A, B, C). *Slit2* is expressed at the midline in the thalamus and strongly at the ventral hypothalamus (Fig. 7H, I, J). The normal expression of these two genes cooperate to prevent TCAs from entering the hypothalamus or crossing the diencephalic midline (Bagri et al, 2002).

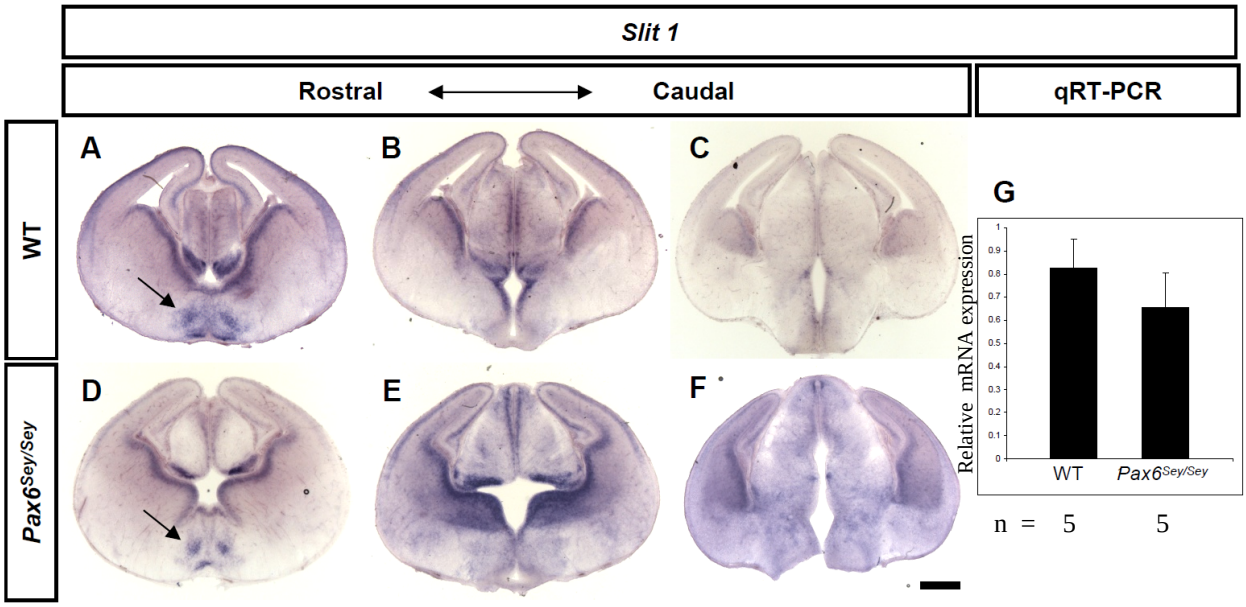
In *Pax6*$^{Sey/Sey}$ mice the expression of *Slit1* can be observed at the midline of the diencephalon and at the hypothalamus (Fig.7D, E, F). *Slit2* expression can be seen strongly at the ventral most region of the hypothalamus (Fig.7K, L, M). To quantify the level of *Slit* expression qRT-PCR was performed on tissue samples of the whole diencephalon dissected from WT and *Pax6*$^{Sey/Sey}$ embryos at E14.5. This analysis showed no significant difference between the level of expression of either *Slit1* or *Slit2* (Fig 7G, N). The fact that expression of these two genes is maintained in the hypothalamus of the *Pax6*$^{Sey/Sey}$ embryo suggests that this region is repulsive to TCAs as in the WT.

Robo1 and *2* are membrane bound proteins that act as receptors for *Slit 1* and *2* (Brose et al., 1999; Kidd et al., 1999). In WT mice at E14.5 *Robo1* mRNA is expressed in the medial thalamus and in the cortical plate (Fig. 8A, B, C). *Robo2* is expressed across the thalamus but absent from the most medial part of the thalamus where *Robo1* expression is strongest. *Robo2* expression is also found at the prethalamus, around the internal capsule in the ventral telencephalon and in the intermediate zone of the cortex (Fig. 8H, I, J). *Robo* expression in thalamic cells with cortical projections ensures that TCAs enter the telencephalon. Knockout studies have shown that in the absence of *Robo2* or both *Robo1* and *2* some TCAs enter the hypothalamus in error (Lopez-Bendito et al, 2007).

In *Pax6*$^{Sey/Sey}$ embryos *Robo1* expression can be seen in a similar position to that seen in the WT although the region of expression appears to be shifted dorsally (Fig. 8D, E, F). The level of *Robo1* expression in the thalamus as determined by qRT-PCR on tissue samples from the whole thalamus is not significantly different between the WT and *Pax6*$^{Sey/Sey}$ (Fig. 8G). The expression pattern of *Robo2* in the *Pax6*$^{Sey/Sey}$ thalamus appears very different from the WT. In the *Pax6*$^{Sey/Sey}$ only a thin line of *Robo2* expression can be seen in the thalamus compared to the large expression domain in the WT (arrow, Fig. 8L). At more caudal levels a larger area of *Robo2* expression can be seen in the thalamus but even here it appears reduced compared to WT (Fig. 8M). *Robo2* expression is also lost in the cortex of the *Pax6*$^{Sey/Sey}$ embryos. The expression found in the ventral telencephalon appears to be maintained, at least at more rostral levels. The level of *Robo2* expression is significantly reduced in the *Pax6*$^{Sey/Sey}$ thalamus when compared to the WT by qRT-

PCR (Student's t-test p = 0.030, WT n = 5, *Pax6*$^{Sey/Sey}$ n = 5) (Fig. 8N). This reduction in *Robo2* expression in the thalamus may explain why TCAs enter the hypothalamus as axons that lack the *Robo* receptor at the growth cone cannot respond appropriately to the *Slit* signal which is present in the hypothalamus.

Figure 7. *Slit* expression is maintained at the hypothalamus in *Pax6*$^{Sey/Sey}$ embryos. **(A-C)** At E14.5 in WT embryos *Slit1* mRNA expression can be seen at the hypothalamus (arrow, A), the prethalamus, the ventricular zone of the ventral telencephalon and the cortical plate. **(D-F)** In *Pax6*$^{Sey/Sey}$ embryos Slit1 expression is still found at the hypothalamus (arrow D). **(G)** qRT-PCR analysis (levels normalised to GAPDH expression) shows no significant difference between the level of *Slit1* mRNA expression in WT and *Pax6*$^{Sey/Sey}$ embryos. **(H-J)** In WT embryos *Slit2* mRNA expression is seen at the hypothalamus (arrows H, I) and the thalamus. **(K-M)** In *Pax6*$^{Sey/Sey}$ embryos *Slit2* expression is still found at the hypothalamus (arrow D). **(N)** qRT-PCR analysis (levels normalised to GAPDH expression) shows no significant difference between the level of *Slit2* mRNA expression in WT and *Pax6*$^{Sey/Sey}$ embryos. Scale bar 500μm.


Slit 1
Rostral
Caudal
qRT-PCR
WT
Pax6Sey/Sey
A
B
C
D
E
F
G
Relative mRNA expression
WT
Pax6Sey/Sey
n = 5 5

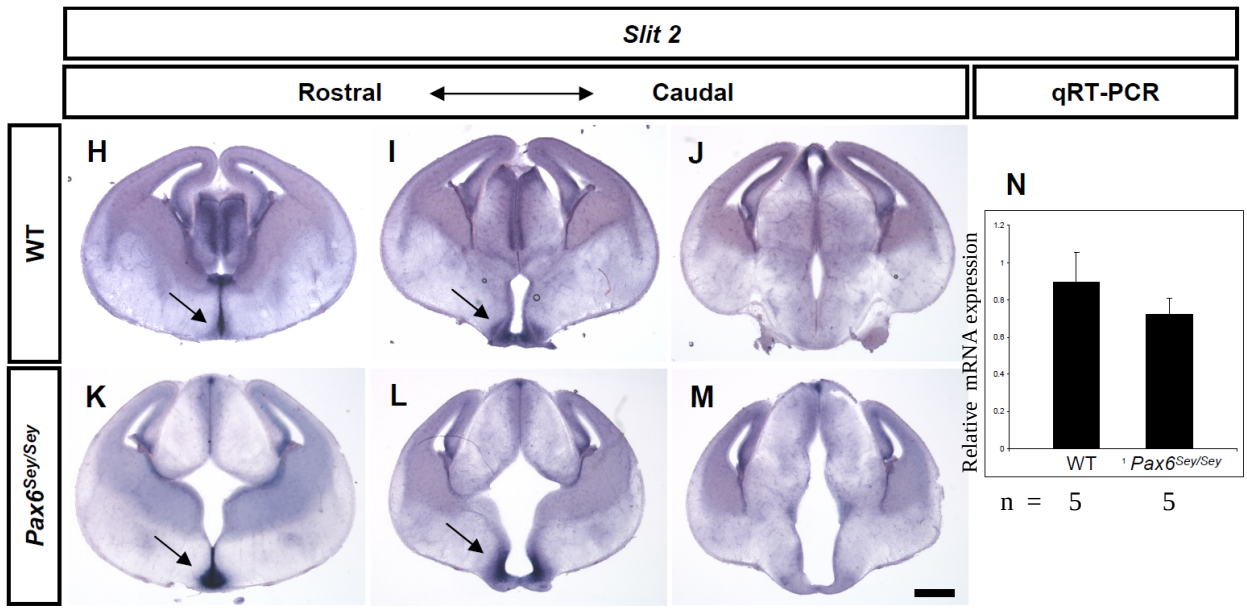
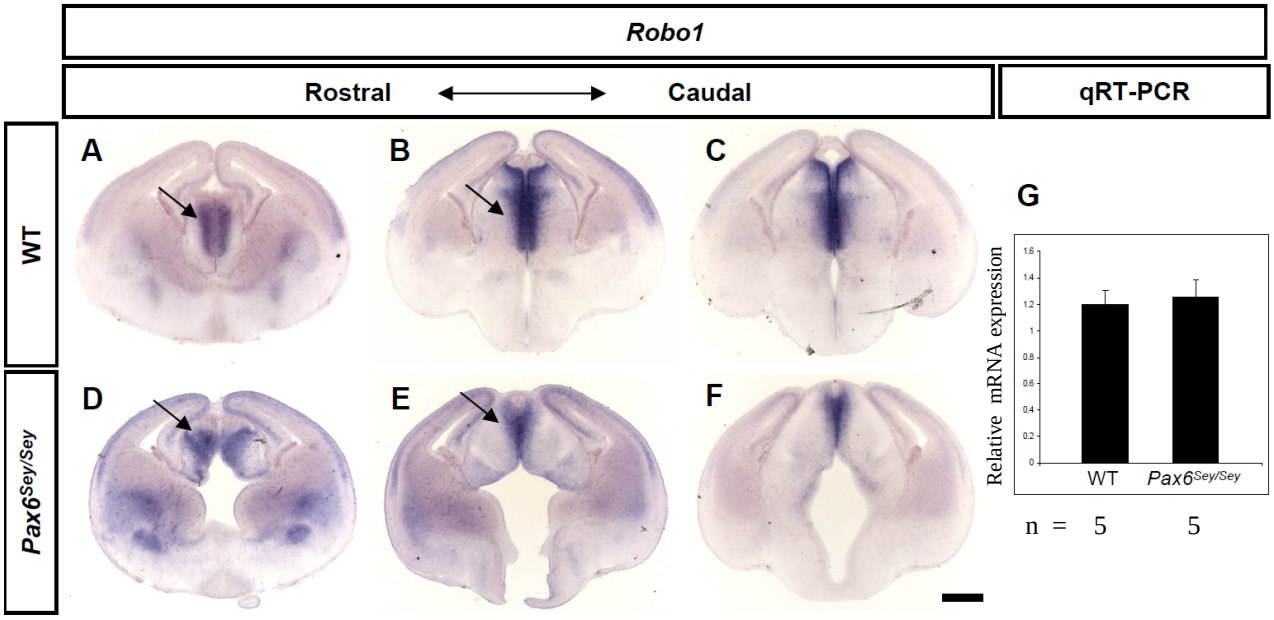
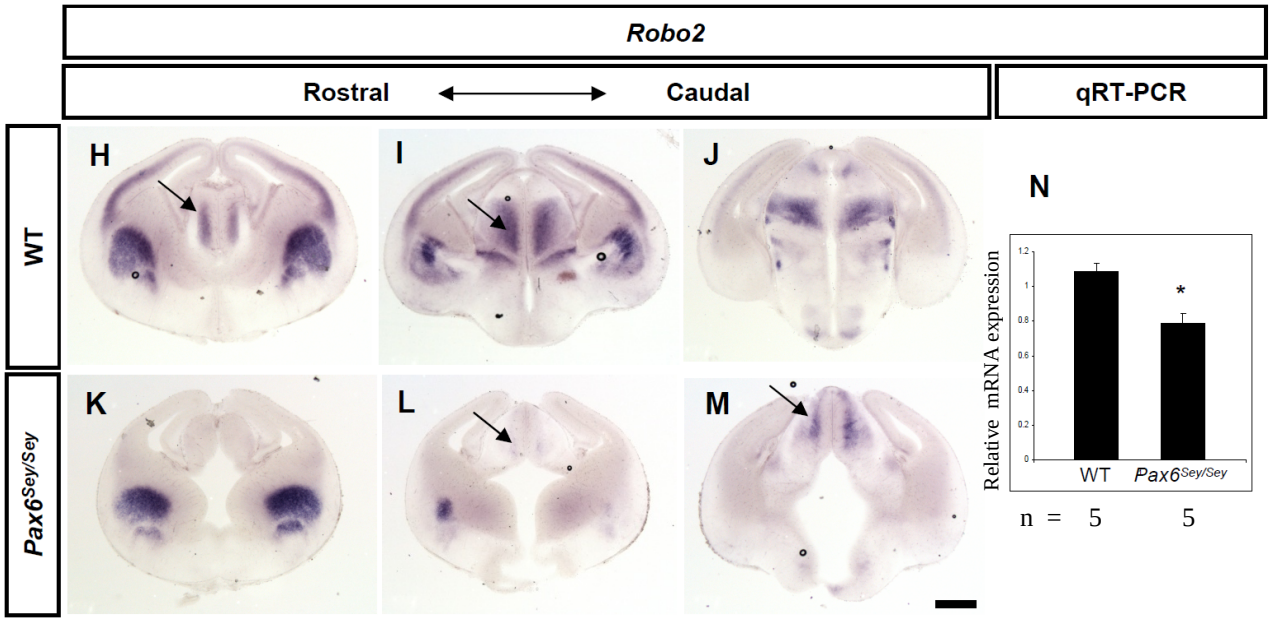

Slit 2
Rostral
Caudal
qRT-PCR
WT
Pax6Sey/Sey
H
I
J
K
L
M
N
Relative mRNA expression
WT
Pax6Sey/Sey
n = 5 5

Figure 8. *Robo2* expression is reduced in the thalamus of *Pax6*$^{Sey/Sey}$ embryos . **(A-C)** At E14.5 in WT embryos *Robo1* mRNA expression can be seen at the thalamus (arrows), the ventral telencephalon and the cortical plate. **(D-F)** In *Pax6*$^{Sey/Sey}$ embryos *Robo1* expression is still found at the thalamus (arrow D). **(G)** qRT-PCR analysis shows no significant difference between the level of *Robo1* mRNA expression in WT and *Pax6*$^{Sey/Sey}$ embryos. **(H-J)** In WT embryos *Robo2* mRNA expression is seen at the thalamus (arrows), the ventral telencephalon and the intermediate zone of the cortex. **(K-M)** In *Pax6*$^{Sey/Sey}$ embryos *Robo2* expression appears markedly reduced in the thalamus. **(N)** qRT-PCR analysis shows a significant reduction in the relative level of *Robo2* mRNA expression in *Pax6*$^{Sey/Sey}$ embryos compared to WT. Scale bar 500μm.


Robo1
Rostral
Caudal
qRT-PCR
WT
Pax6Sey/Sey
A
B
C
D
E
F
G
Relative mRNA expression
WT
Pax6Sey/Sey
n = 5 5


Robo2
Rostral
Caudal
qRT-PCR
WT
Pax6Sey/Sey
H
I
J
K
L
M
N
Relative mRNA expression
*
WT
Pax6Sey/Sey
n = 5 5

3.3 Discussion

3.3.1 Summary

In this chapter we have seen that in embryos which lack functional *Pax6* expression the thalamocortical tract fails to form. Contrary to previous findings (Jones et al., 2002) it was observed that TCAs do not reach the telencephalon in *Pax6* null embryos and that the *L1* positive axon bundle present in the ventral telencephalon of these animals is of telencephalic rather than thalamic origin. The cell type from which this tract is derived remains unclear. In line with previous findings from Simpson et al., 2009 experiments in this chapter show that the axon permissive 'corridor' which guides TCAs through the ventral telencephalon (Lopez-Bendito et al., 2006) is malformed in *Pax6* null embryos. This further underlines the importance of *Pax6* expression for the development this important TCA guidance mechanism. Examination of the expression of *Slit* guidance molecules and their receptors *Robo* in *Pax6* null embryos has shown that while Slit expression is largely maintained *Robo2* expression within the thalamus is significantly reduced. This suggests that *Pax6* promotes *Robo2* expression within the thalamus and provides further evidence that *Pax6* influences axon guidance by regulating the expression of axon guidance cues.

3.3.2 Thalamocortical axons are misrouted to the hypothalamus and do not reach the ventral telencephalon in the *Pax6*$^{Sey/Sey}$ mouse

The axon tracing experiments described in this chapter indicate that TCAs do not reach the cortex in the *Pax6*$^{Sey/Sey}$ mouse as they do in the WT. Indeed TCAs do not even cross the DTB to reach the ventral telencephalon. A small number of axons have been shown to leave the thalamus but these axons do not make the required lateral turn towards the telencephalon and instead head towards the hypothalamus. Immunohistochemistry for axonal marker L1 has shown that a large bundle of TCAs are produced by cells within the thalamus but it appears that the majority of these axons cannot leave the thalamus. This finding is broadly consistent with other studies

of the thalamocortical tract in rodents that lack *Pax6* (Hevner et al., 2002; Jones et al., 2002; Pratt et al., 2002)

There are several possible reasons why a large number of the TCAs in the thalamus are not able to leave. Firstly it may be due to a purely mechanical problem arising from the fact that at more rostral levels the dorsal part of the diencephalon is not anatomically connected to the ventral telencephalon in the *Pax6*$^{Sey/Sey}$ mouse. This feature is particularly prominent prior to E14.5 at which time in the WT TCAs leave the thalamus and are guided into the ventral telencephalon. After E14.5 the diencephalon is connected to the telencephalon along its rostro-caudal axis but this delay could impact on the development of the tract. Another possibility is that the pioneer axon tracts that guide TCAs are absent or malformed in the *Pax6*$^{Sey/Sey}$ mouse as with *Mash1* mutant mice (Tuttle et al., 1999). Evidence from the *Pax6*$^{LacZ/KO}$ mouse suggests that at least some pioneer axons that extend from the internal capsule zone are present, and those that originate from the reticular nucleus of the prethalamus are displaced ventrally (Jones et al., 2002). Given the morphological defects seen in this region it is highly likely that these pioneer axons will be disturbed. It is also possible that there are changes in the expression of key guidance molecules in the *Pax6*$^{Sey/Sey}$ mouse, either on the growth cone of the axon or in the environment through which the axon is guided.

For TCAs to cross the DTB and enter the telencephalon, cells expressing the membrane bound form of the guidance cue *Neuregulin1* must migrate tangentially from the LGE to form an axon permissive 'corridor' within the MGE through which TCAs can grow (Lopez-Bendito et al., 2006). Examination of markers for the corridor cells and the MGE show that the corridor is severely malformed in *Pax6*$^{Sey/Sey}$ embryos; this is likely to greatly impair the ability of TCAs to reach the ventral telencephalon. The repulsive MGE also appears expanded, with repulsive guidance cue *Slit1* expressed throughout this expanded region. This may repel TCAs from the telencephalon, preventing TCAs from crossing the DTB. This finding provides further evidence that *Pax6* plays a crucial role in the development of the corridor. Simpson et al (2009) found that when *Pax6* expression was specifically reduced in cells close to the amygdaloid region the corridor became abnormally widened. They suggested that *Pax6* acted in these cells to restrict the migration of the

cells from the LGE to ensure a narrow corridor capable of channelling TCAs towards the cortex. The phenotype of *Pax6*$^{Sey/Sey}$ embryos is a much more severe than that seen in the conditional mutant analysed by Simpson et al, which is to be expected due to the fact that *Pax6* expression is absent throughout the ventral telencephalon. What is unclear is the degree to which *Pax6* expression is required within the progenitors of cells that will form the corridor. In chapter four, conditional mutagenesis has been used in to delete *Pax6* expression within these cells in an attempt to answer this question.

3.3.3 *Slit* expression is maintained in the hypothalamus of *Pax6*$^{Sey/Sey}$ embryos while *Robo2* expression is reduced in the thalamus.

Previous work has suggested *Pax6* may play a role in regulating the expression of molecular guidance cues (Jones et al., 2002; Tsuchiya et al., 2009). In this chapter the expression of *Slit* and *Robo* guidance cues was analysed in the *Pax6*$^{Sey/Sey}$ mouse. It was found that the expression of *Slit1* and *2* was maintained in the hypothalamus while *Robo2* expression was reduced within the thalamus. This finding could help explain the why TCAs enter the hypothalamus in the *Pax6*$^{Sey/Sey}$ mouse unlike in the WT where TCAs are repelled from this region. A reduction in the expression of *Robo2* at the growth cone would reduce the sensitivity of the axon to the repulsive *Slits* expressed at the hypothalamus, which could lead to TCAs invading the hypothalamus rather than turning laterally towards the telencephalon.

Is this reduction in *Robo2* expression sufficient to explain the axonal phenotype seen in the *Pax6*$^{Sey/Sey}$ mouse? In mice where *Robo2* is knocked out a number of TCAs enter the hypothalamus which is consistent with what is seen in the *Pax6*$^{Sey/Sey}$ mouse. The phenotype of this knockout is however, much less severe as a large number of TCAs do reach the cortex normally, and in addition the morphology of the brain is much less distorted than in the *Pax6*$^{Sey/Sey}$ (Lopez-Bendito et al., 2007). This suggests that the loss of *Robo2* is at least partly responsible for the phenotype seen in the *Pax6*$^{Sey/Sey}$ mouse, but the severity of the phenotype means that this is unlikely to be the only cause. It is likely that a number of factors including the reduction in *Robo2* expression, the disruption to the axon permissive corridor within the ventral telencephalon, morphological changes and disruption of pioneer axon

tracts combine to cause the axon path-finding defects observed in the *Pax6*$^{Sey/Sey}$ mouse.

This fact that *Robo2* expression is reduced in the thalamus of mice that lack *Pax6,* in addition to previously observed changes in the expression of *Semaphorins* (Jones et al., 2002) and *Netrin1* (Tsuchiya et al., 2009) suggest that *Pax6* can influence axon guidance by regulating the expression of diffusible guidance cues or their receptors. What remains uncear is whether *Pax6* is able to directly modulate the transcription of *Robo2* and other guidance cues.

3.3.4 Aberrant axon tract found within the *Pax6*$^{Sey/Sey}$ ventral telencephalon

In this chapter a large L1 positive bundle of axons has been observed within the ventral telencephalon of the *Pax6*$^{Sey/Sey}$ mouse between E14.5 and E18.5. This axon tract has been observed by previous studies, either by L1 immunohistochemistry (Jones et al., 2002) or by DiI placement within the ventral telencephalon (Kawano et al., 1999). The tract has been assumed to be made up of TCAs but in these studies the tract has not been labelled following DiI placement at the thalamus which would be necessary to prove the tract was of thalamic origin. DiI labelled axons have been described within the ventral telencephalon following thalamic DiI placement (Hevner et al., 2002) but these axons are small in number and spread out in contrast to the large tight bundle observed here.

In this chapter it is shown conclusively that this axon tract is not of thalamic origin. Firstly DiI placement at the ventral aspect of the ventral telencephalon labels the tract and crucially cell bodies close to the PSPB but not at the thalamus. Secondly DiI placement close to the PSPB labels the axons of the tract including growth cone tipped axons adjacent to the amygdaloid region and once again no label is seen in the thalamus. This allows us to conclude that this tract is not made up of TCAs but rather the axons originate from cells within the ventral telencephalon itself and extend ventrally towards the base of the brain.

If the axons observed in this tract are not of thalamic origin but instead arise from cells in the ventral telencephalon the obvious question to ask is what these cells are. One possibility is that they are cortical cells that are displaced and the axons that

they extend are corticothalamic or corticospinal. This, however, is unlikely. Previous studies have described how the telencephalon of *Pax6*$^{Sey/Sey}$ mice display a ventralisation i.e. markers of ventral telencephalic cells (e.g. *Mash1*) are shifted dorsally and dorsal markers (e.g. *Ngn2*) are shifted further dorsally (Stoykova et al., 2000). Thus it would be expected that cortical cells with axonal projections are more likely to be displaced dorsally rather than ventrally. Another possibility is that these axons could be from the various short range local axonal connections that form linking the developing regions of the basal ganglia and amygdala (Ottersen, 1980). These axonal connections however seem to be too few to form the large tract seen in the *Pax6*$^{Sey/Sey}$ and no tract was labelled by the DiI placement at the ventral telencephalon of the WT. Another possibility is that these cells are interneurons. GABAergic interneurons migrate tangentially into the cortex and the olfactory bulb from the MGE and to a lesser extent the LGE (Marin and Rubenstein, 2001); however neuronal migration is disturbed in the *Pax6*$^{Sey/Sey}$ mouse (Caric et al., 1997) and these GABAergic internurons form ectopias in both the dorsal and ventral telencephalon (Kroll and O'Leary, 2005). It is possible that the axons in question may have arisen from a population of interneurons that have been unable to migrate to the cortex.

In order to ascertain the nature of the cells from which the axon tract originates further work would have to be undertaken. The examination of the expression of a variety of molecular markers within the ventral telencephalon would give a greater understanding of what cells are present in this region. If this could be combined with carbocyanine dye tracing then it would be possible to determine exactly what markers are expressed by the cells which contribute axons to this tract. Molecular markers that might be used include markers for interneurons such as *Dlx1/2* and markers of postmitotic cells found in the region including *Islet1* and *Lhx6*.

Chapter 4: The role of Pax6 at the prethalamus and ventral telencephalon for thalamocortical development

4.1 Introduction

In the previous chapter the small eye (*Pax6*$^{Sey/Sey}$) mouse was used as a model to understand the role that *Pax6* plays in the formation of the thalamocortical tract. This mutant mouse, which completely lacks functional *Pax6*, has been very useful in shaping our understanding of the function of this gene. The identification of a role for *Pax6* in the regulation of proliferation, neuronal patterning, cell migration and axon guidance have all come from use of the *Pax6*$^{Sey/Sey}$ mouse (Caric et al., 1997; Mastick et al., 1997; Stoykova et al., 2000; Warren et al., 1999).

There are, however, disadvantages to using this mouse model, and other complete loss-of-function mutant mice such as the *Pax6 LacZ* knockout. Because of the wide range of developmental processes in which *Pax6* is involved, the complete absence of *Pax6* is lethal post-natally. In addition the severity of the embryonic phenotype can make it difficult to interpret the cause of any abnormalities observed in the *Pax6*$^{Sey/Sey}$ mouse. It may be impossible to determine whether a particular phenotype is caused primarily by the lack of *Pax6* or is secondary to another defect caused by the loss of the gene. This issue can be of particular concern when studying axon guidance as the profound morphological defects seen in the *Pax6*$^{Sey/Sey}$ mouse are likely to have a great influence on the guidance of axons.

In order to circumvent these problems two different approaches have been used. Chimeric mice have been created containing a mixture of wild type and *Pax6*$^{Sey/Sey}$ cells; this model will be discussed in more detail in chapter 5. *Cre-lox* technology can be used to knock out gene function within specific tissues while maintaining normal gene expression elsewhere in the animal. This involves the combination of a mouse which expresses *Cre recombinase* under the influence of a region specific promoter with another mouse which has *loxP* sites inserted flanking one or more exons of the target gene. The *Pax6*$^{flox/flox}$ mouse has *loxP* sites flanking exons 5, 5a and 6 of the *Pax6* gene which encode the paired DNA binding domain essential for *Pax6* function (Simpson et al., 2009). Upon exposure to the *Cre recombinase* enzyme this region is excised and *Pax6* is rendered non-functional.

The *Pax6*$^{flox/flox}$ mouse has been combined with a number of different *Cre* lines. The *Emx1 Cre* has been used to create a cortex specific Pax6 knockout. This

mouse has been used to demonstrate that *Pax6* expression within the cortex is essential for the correct formation of the cortical layers (Georgala et al., 2011; Tuoc et al., 2009). The TCAs of these mice are able to reach the cortex normally, and the topographic arrangement of the thalamocortical tract is also maintained. This demonstrates that *Pax6* expression within the cortex is not required for TCAs to reach the cortex correctly (Pinon et al., 2008).

The *Six3 Cre* has also been used to create a *Pax6* conditional knockout. This mouse has reduced *Pax6* expression among a population of cells within the ventral telencephalon close to the amygdaloid region. This mouse displays both thalamocortical and corticothalamic axon path-finding defects. A small number of TCAs do not cross the DTB and are misrouted ventrally; in addition some TCAs within the ventral telencephalon turn ventrally when passing through the internal capsule zone. It was determined that the *Islet1* positive 'corridor' within the MGE which is responsible for guiding axons through this region (Lopez-Bendito et al., 2006) is disturbed in this mutant mouse which may be responsible for the TCA path-finding defects that have been observed (Simpson et al., 2009). This study has demonstrated that *Pax6* expression is required at this region for the correct formation of the thalamocortical tract, it also suggests that *Pax6* plays a role in the formation of the permissive 'corridor' within the MGE through which TCAs are guided.

In this chapter the *Gsh2 Cre* mouse line (Kessaris et al., 2006) has been used to create a *Pax6* conditional knockout mouse ($Pax6^{cKO}$) in which *Pax6* expression is specifically reduced in the prethalamus and the ventral telencephalon. This mouse has then been used to investigate the role that *Pax6* expression at these locations plays in the development of the thalamocortical tract. Previous work has shown that this mouse displays patterning defects across the PSPB and altered neuronal diversity within the amygdala (Cocas et al., 2011). Here, tract tracing and immunohistochemistry has been used to examine the thalamocortical tract of the $Pax6^{cKO}$ mouse. Immunohistochemistry has also been used to analyse the development of the pioneer axons which provide guidance for TCAs. In light of the patterning defects observed at the PSPB, *in situ* hybridisation and immunohistochemistry has been used to examine the patterning of the prethalamus.

4.2 Results

4.2.1 Creation of a Pax6 conditional knockout mouse

We have seen that a complete loss of *Pax6* from the mouse causes severe defects in the formation of the thalamocortical tract. These observed axon path finding defects are in addition to major morphological changes that can make it difficult to define the precise role that *Pax6* plays in thalamocortical axon guidance. In an effort to counter this problem conditional mutagenesis has been used to specifically knockout *Pax6* in *Gsh2* derived cells within the prethalamus and the LGE of the ventral telencephalon, two regions through which TCAs navigate during embryogenesis. To do this the $Pax6^{flox/flox}$ mouse was used which has *loxP* sites flanking exons 5, 5a and 6 which encode the paired DNA binding domain (Simpson et al., 2009). The $Gsh2^{Cre}$ mouse was then used to drive Cre-recombination in the *Gsh2* expressing cells found within the prethalamus and ventral telencephalon (Kessaris et al., 2006). The RCE EGFP reporter (Sousa et al., 2009) was also used to identify cells where Cre recombinase was active. *Gsh2* expression within the mouse begins at around E10.0 and is found at the ventricular zone of both the LGE and the prethalamus (Corbin et al., 2003) and *Cre* recombinase activity can be observed in the $Gsh2^{Cre}$ mouse by E11.5 (Cocas et al., 2011). The genotype of the conditional knockout created was $Gsh2Cre^{+/-}$ $Pax6^{flox/flox}$ RCE $EGFP^{+/-}$ (referred to as the $Pax6^{cKO}$ mouse) and the control was $Gsh2Cre^{+/-}$ $Pax6^{+/+}$ RCE $EGFP^{+/-}$ (referred to as control).

Immunohistochemistry for GFP shows where Cre recombinase is active. At E14.5 GFP expression can be seen throughout much of the ventral telencephalon and the prethalamus. These cells either express *Gsh2* or are from a lineage of *Gsh2* expressing cells. Immunohistochemistry for axonal marker *L1* indicates the position of the thalamocortical tract which passes through both of these regions *en route* to the cortex (Fig. 1C).

In order to determine if *Pax6* expression was indeed knocked out in the LGE and the prethalamus of the $Pax6^{cKO}$ mouse immunohistochemistry for *Pax6* was carried out at E14.5. By this stage *Cre* recombinase has been active for

approximately 3 days and the floxed region within the *Pax6* gene should have been excised. The epitope recognised by the *Pax6* antibody is located within the floxed region of the *Pax6* protein which means that only functional *Pax6* protein will be detected.

In the control *Pax6* expression can be seen within the prethalamus, the epithalamus and faintly at the ventricular zone of the thalamus. Expression can also be seen within the ventricular zone of the developing cortex and extending along the ventricular zone of the LGE. There are also Pax6 expressing cells found at the amygdaloid region within the ventral telencephalon (Fig. 1A) (Stoykova and Gruss, 1994). In the $Pax6^{cKO}$ *Pax6* expression is maintained at the epithalamus, the thalamus and at the ventricular zone of the cortex (Fig 1B). Within the ventral telencephalon *Pax6* expression is reduced at the amygdaloid region (Fig. 1B) (Cocas et al., 2011). Pax6 expression is also lost from the ventricular zone of the LGE where the Pax6 expression domain stops abruptly close to the PSPB rather than the lateral-high to medial-low gradient seen in the control (arrows Fig 1D, E). In the diencephalon it can be seen that *Pax6* expression is reduced in the prethalamus of the $Pax6^{cKO}$ compared to control (Fig. 1F, G), the number of *Pax6* positive cells in the prethalamus is significantly reduced in the $Pax6^{cKO}$ (Students t test p= 0.031, control n= 4, $Pax6^{cKO}$ n= 4) (Fig. 1H). Fluorescent immunohistochemistry for *Pax6* and GFP shows a population of cells within the control prethalamus that are double labelled (arrow Fig. 1G). These cells both express *Pax6* and show *Cre* recombinase activity. In the $Pax6^{cKO}$ however this population of double labelled cells is absent (arrow Fig 1H) indicating that *Pax6* expression has been reduced in cells where *Cre* is active.

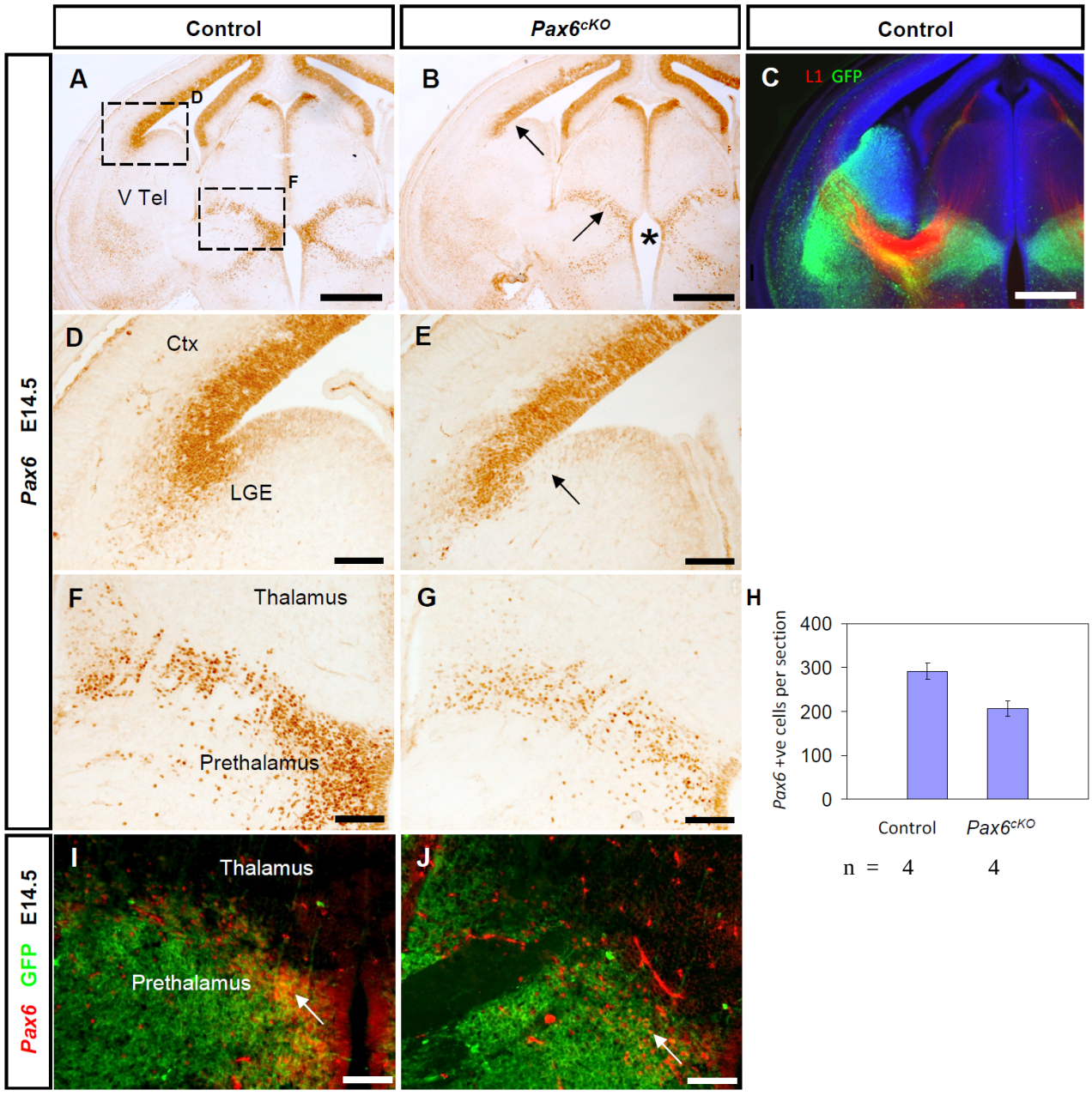
Morphologically control and $Pax6^{cKO}$ embryos are identical with the exception that in approximately 1/3 of cases the third ventricle is slightly enlarged in the $Pax6^{cKO}$ embryo compared to that in the control (asterisk Fig. 1B, Fig. 12H, J).

Table 1. A breakdown of the number of animals used in each of the experiments conducted as part of this chapter.

			n number	
Figure	Experiment	Age	Control	*Pax6*^{cKO}
1	*Pax6* immunohistochemiostry (DAB)	E14.5	4	4
	Pax6/GFP immunohistochemistry (fluorescent)	E14.5	2	2
	L1/GFP immunohistochemistry (fluorescent)	E14.5	2	0
2	Thalamic DiI placement	E14.5 E16.5	3 3	5 3
3	Cortical DiI placement	E14.5 E16.5	3 3	3 3
4 & 5	*L1* Immunohistochemistry	E14.5 E16.5 E18.5	3 2 2	5 3 3
6 & 7	Cortical DiI/DiA placement	E18.5	4	4
8 & 9	GFP immunohistochemistry	E12.5 E13.5 E14.5	3 3 3	3 3 3
10	GFP immunohistochemistry on DTy54 cross	E14.5	3	3
11	Cleaved-*Caspase3* immunohistochemistry	E13.5	3	3
12	*Ngn2 in situ* hybridisation	E13.5	2	2
	Shh in situ hybridisation	E13.5	2	3
	Lim1/2 immunohistochemistry	E13.5	2	2
	Islet1 immunohistochemistry	E13.5	3	3
	Mash1 immunohistochemistry	E13.5	2	2
	Nkx2.2 immunohistochemistry	E13.5	2	2

	R-Cad immunohistochemistry	E13.5	2	2
13	*Dbx1 in situ* hybridisation	E13.5	2	3
	Ngn2 in situ hybridisation	E13.5	2	2
	Islet1 immunohistochemistry	E13.5	3	3

Figure 1. Reduction of *Pax6* expression within the LGE and prethalamus of the *Gsh2Cre* conditional knockout (*Pax6*^{cKO}). (A, B, D-F) *Pax6* immunohistochemistry shows a reduction in *Pax6* expression at E14.5 at the ventricular zone of the LGE (arrows, E) and the prethalamus (G) of the *Pax6*^{cKO} compared to the control (D, F). **(C)** Immunohistochemistry for GFP shows that Cre is active in cells of the prethalamus and ventral telencephalon, *L1* immunohistochemistry is used to visualise the position of the thalamocortical tract. **(I, J)** Immunohistochemistry shows *Pax6* and GFP expression within the prethalamus. An area of yellow double labelled cells can be seen in the control (arrow, G) but not in the *Pax6*^{cKO} (arrow, H). **(H)** Quantification of the density of *Pax6* expressing cells within the control and *Pax6*^{cKO} prethalamus. Scale bars: 500μm in A-C,100μm in D-J.


Control
Pax6cKO
Control
Pax6 E14.5
A
D
V Tel
F
B
*
C
L1 GFP
D
Ctx
LGE
E
F
Thalamus
Prethalamus
G
H
Pax6 +ve cells per section
400
300
200
100
0
Control
Pax6cKO
n = 4 4
Pax6 GFP E14.5
I
Thalamus
Prethalamus
J

4.2.2 Thalamocortical axons can cross the PSPB and reach the cortex in the *Pax6^{cKO}* embryo

In order to investigate what effect the reduction in *Pax6* expression has on the development of the thalamocortical tract DiI was injected into the thalamus of *Pax6^{cKO}* and control embryos at E14.5 and E16.5 to label the TCAs. In the control at E14.5 DiI labelled TCAs can be seen leaving the thalamus turning laterally into the telencephalon and have extended as far as the PSPB (Fig. 2A). In the *Pax6^{cKO}* TCAs can also be seen leaving the thalamus and extending as far as the PSPB however as the TCAs cross through the prethalamus they form a narrower bundle than in the control (control n= 3, *Pax6^{cKO}* n= 5) (arrow Fig. 2B). By E16.5 in the control TCAs have crossed the PSPB and reached the cortex (Fig. 2C, E). At this stage corticothalamic axons (CTAs) have not yet reached the thalamus (De Carlos and O'Leary, 1992) so all the labelled axons must be TCAs. In the *Pax6^{cKO}* TCAs can be seen crossing the PSPB as they do in the control (control n= 3, *Pax6^{cKO}* n= 3) (Fig. 2F). This shows that despite the loss of *Pax6* at the prethalamus and the LGE TCAs are still able to cross the PSPB and reach the cortex although it appears that the guidance of TCAs is affected as the axons navigate through the prethalamus.

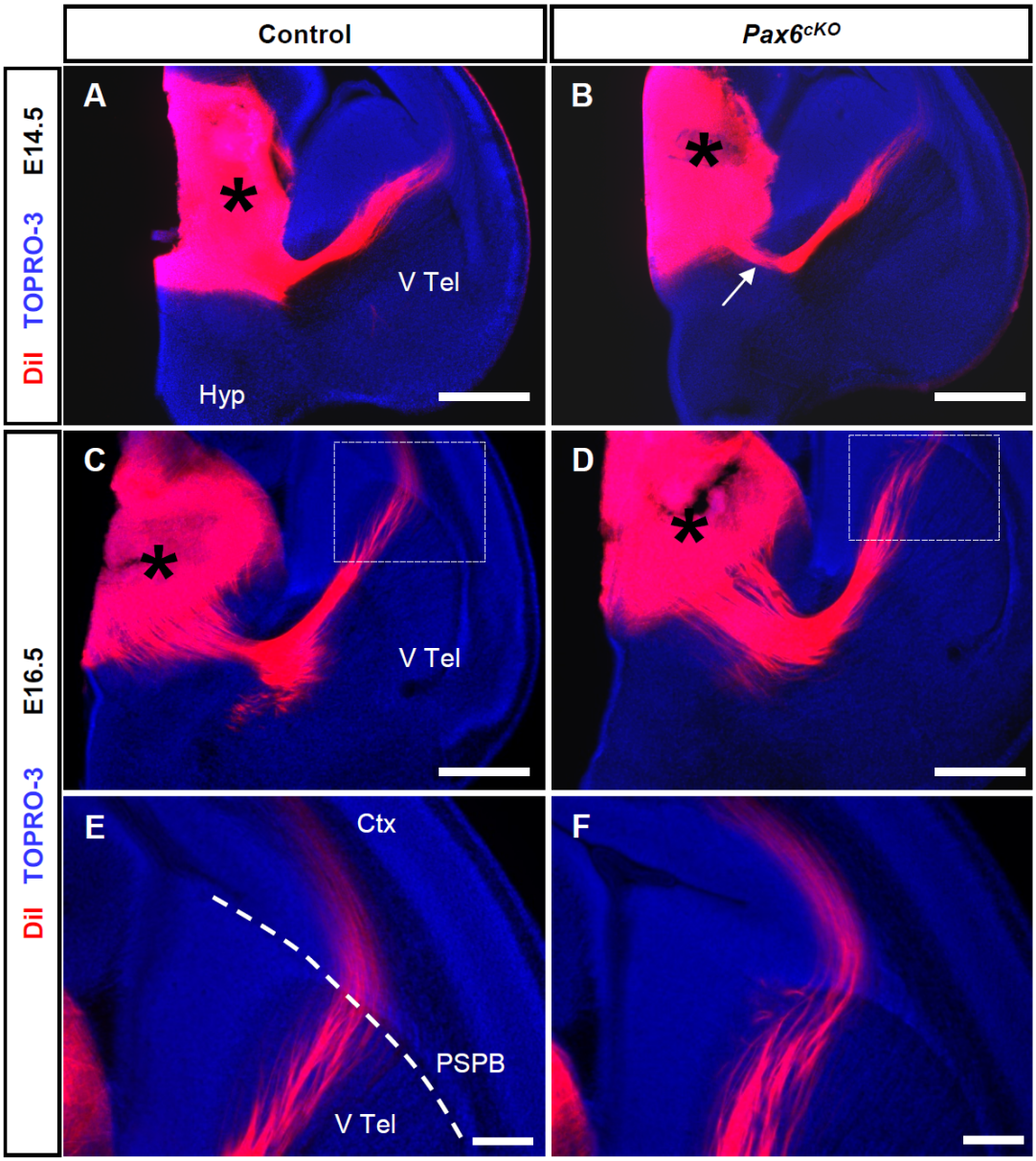


Figure 2. **Thalamocortical axons reach the ventral telencephalon normally and cross the PSPB in the *Pax6*^{cKO}. (A,B)** DiI placement in the thalamus (asterisk) reveals thalamocortical axons extending into the ventral telencephalon reaching the PSPB at E14.5 in both control and *Pax6*^{cKO}. **(C,D,E,F)** At E16.5 axons can be seen crossing the PSPB (dashed line) to reach the cortex normally in both the control and *Pax6*^{cKO} (E, F). E and F are higher magnification images of the boxed regions in A and B respectively. Scale bars: 500µm in A-D, 100µm in E and F.

4.2.3 Corticothalamic axons cross the PSPB and reach the ventral telencephalon normally in *Pax6^{cKO}* embryos

To investigate the behaviour of CTAs in the *Pax6^{cKO}* mouse DiI was placed in the cortex at E14.5 and E16.5. In the control at E14.5 a bundle of DiI labelled CTAs can be seen leaving the cortex and entering the ventral telencephalon (Fig. 3A). A number of these axons can be seen crossing the PSPB (Fig. 3C). This is also the case in the *Pax6^{cKO}* where axons have crossed the PSPB and entered the ventral telencephalon (Fig. 3B, D). It appears that the loss of *Pax6* in the LGE does not affect the ability of CTAs to reach the ventral telencephalon.

By E16.5 TCAs have reached the cortex (Fig. 2) (Auladell et al., 2000) and so DiI placement in the cortex labels both TCAs and CTAs. In both the control and the *Pax6^{cKO}* DiI diffusion has labelled TCAs and CTAs crossing the PSPB (Fig. 3E-H). DiI labelling also reveals the 'fan-like' arrangement of axons seen in the internal capsule (Molnar et al., 1998a). In both the control and the *Pax6^{cKO}* this structure can be observed (arrows Fig. 3E, F). In both the control and the *Pax6^{cKO}* cell bodies have been labelled within the thalamus by retrograde DiI diffusion along TCAs (Fig. 3F, H). This is consistent with the data from Fig. 1 showing that TCAs reach the cortex in the *Pax6^{cKO}* mouse.

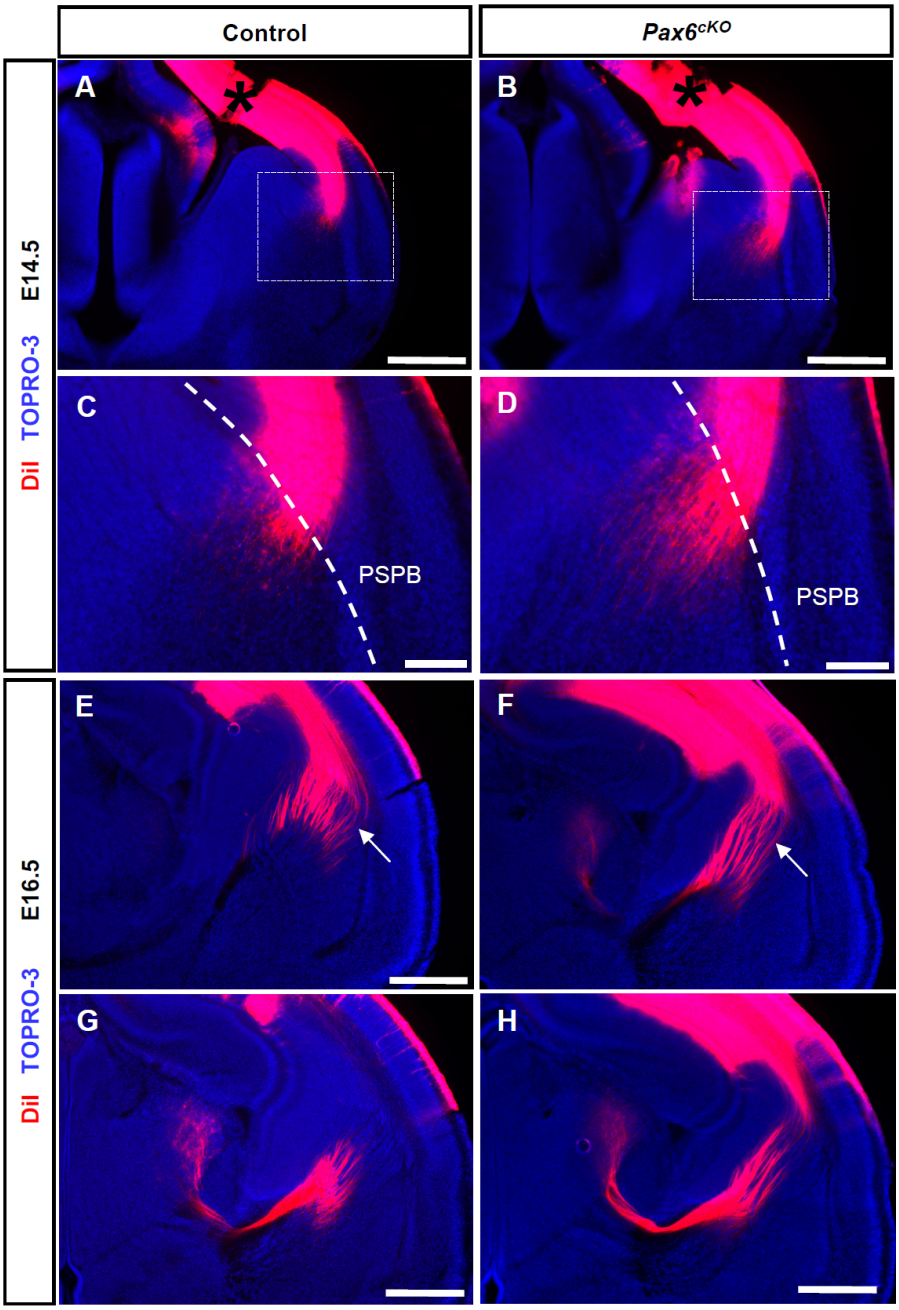


***Figure* 3. Corticothalamic axons cross the PSPB to reach the ventral telencephalon normally in the *Pax6*^{cKO} mouse. (A-D)** DiI placement in the cortex (asterisk) at E14.5 labels corticothalamic axons crossing the PSPB in both the control (A,C) and the *Pax6*^{cKO} (B,D). **(E-H)** DiI placement in the cortex at E16.5 labels both corticothalamic and thalamocortical axons within the internal capsule in both the control and *Pax6*^{cKO} (arrows E,F). Sections rostral to E and F show DiI labelled cell bodies in the thalamus (G,H). C and D are higher magnification images of the boxed regions in A and B respectively. Scale bars: 500μm in A,B,E-H, 100μm in C and D.

4.2.4 Thalamocortical axons appear disorganised within the thalamus of *Pax6^{cKO}* embryos

To further examine the thalamocortical tract of the *Pax6^{cKO}* mouse, L1 immunohistochemistry was performed at E14.5, E16.5 and E18.5. This allows the labelling of the entire thalamocortical tract throughout its development. In addition this approach allows the visualisation of TCAs within the thalamus, which is difficult using thalamic DiI placement.

At E14.5 in the control TCAs can be seen leaving the thalamus in a broad smooth curve, and turning towards the telencephalon (Fig. 4A). In the *Pax6^{cKO}* TCAs can also be seen leaving the thalamus and turning towards the telencephalon (Fig. 4B), this is consistent with what was observed in the DiI tract tracing experiment. At E16.5 in the control, TCAs can be seen once again leaving the thalamus in a smooth curve as they head towards the telencephalon (Fig. 4C). In the *Pax6^{cKO}* however the TCAs appear more highly fasciculated as they cross the prethalamus (Fig. 4D). At E16.5 and E18.5 axons can be seen crossing the PSPB in both the control and the *Pax6^{cKO}*. The 'fan-like' arrangement of axons within the internal capsule, previously observed by cortical DiI placement, can also be seen in both genotypes (Fig. 4C-F).

Looking at the higher magnification images it can be seen that in the *Pax6^{cKO}* at E14.5 TCAs appear disorganised within the thalamus. TCAs display a 'frayed' appearance (arrow Fig. 5B) compared to the smooth curve of axons in the control (Fig. 5A). Some TCAs in the *Pax6^{cKO}* form bundles which project laterally rather than ventrally (n =5). (arrowhead Fig. 5B). The disorganised appearance of TCAs within the thalamus of the *Pax6^{cKO}* is maintained later in embryonic development as it is seen at both E16.5 (*n* =3) and E18.5 (*n* =3) (arrows Fig. 5D, F). In 100% of the *Pax6^{cKO}* embryos analysed at E14.5, E16.5 and E18.5 both the disorganisation of TCAs within the prethalamus and abnormal axon bundles within the thalamus were observed compared to 0% of control embryos. It is curious that TCAs are affected within the thalamus as *Pax6* expression is unaffected in this region, unlike the prethalamus where *Pax6* expression is reduced. This suggests that the prethalamus is involved in guiding TCAs from the thalamus and that *Pax6* expression is required for this process to occur.

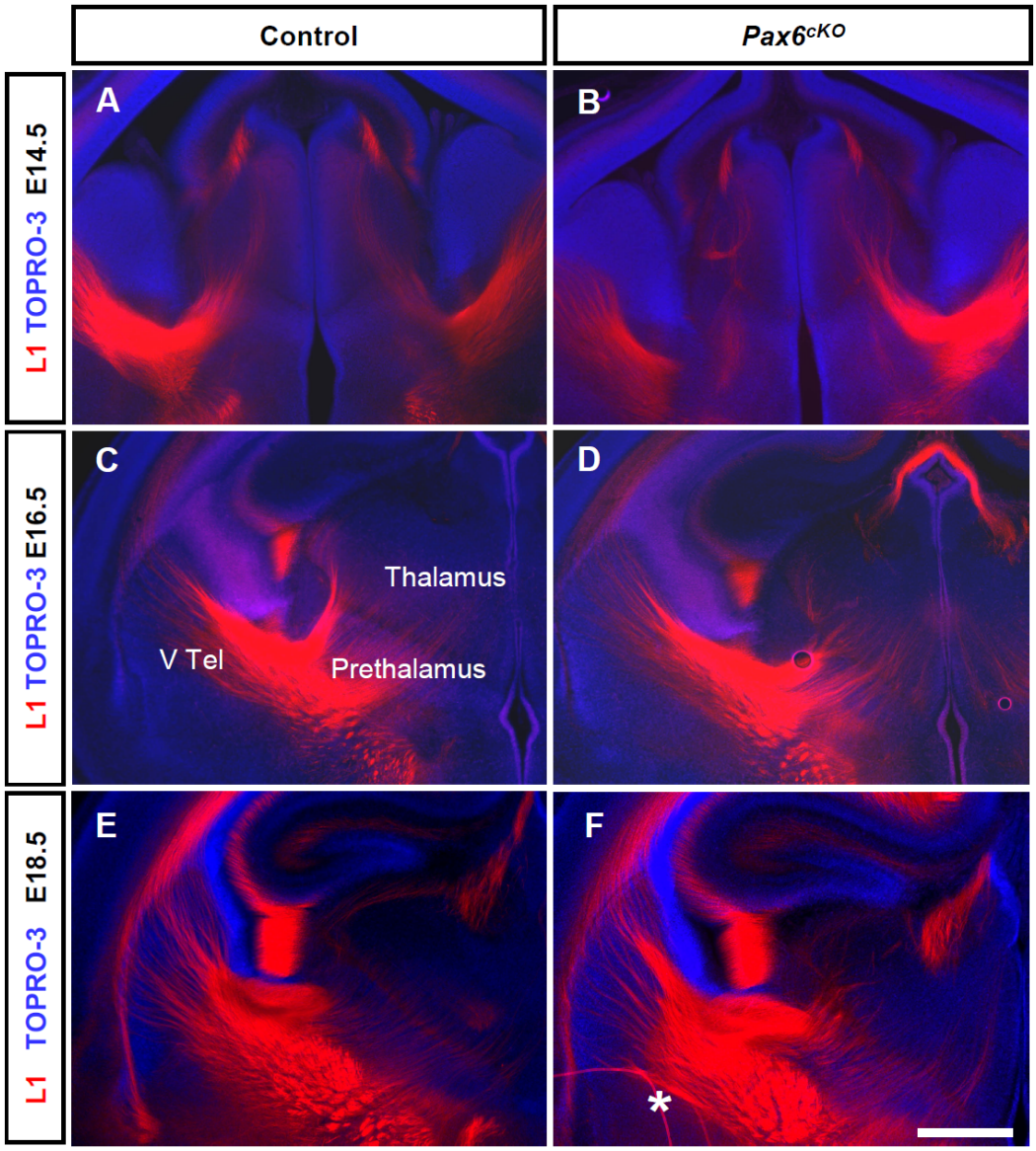


Figure 4. Immunohistochemistry for axonal marker L1 shows thalamocortical axons reach the cortex and form the internal capsule normally in *Pax6*^{cKO} embryos. *L1* Immunohistochemistry at E14.5 (A, B), E16.5 (C, D) and E18.5 reveal the TCAs of the thalamocortical tract. TCAs reach the cortex in both the control (A, C, E) and the *Pax6*^{cKO} (B, D, F) In both genotypes the fan-like structure of the internal capsule can be observed at E16.5 (C, D) and E18.5 (E, F). Asterisk indicates tissue artefact. Scale bar: 500µm.

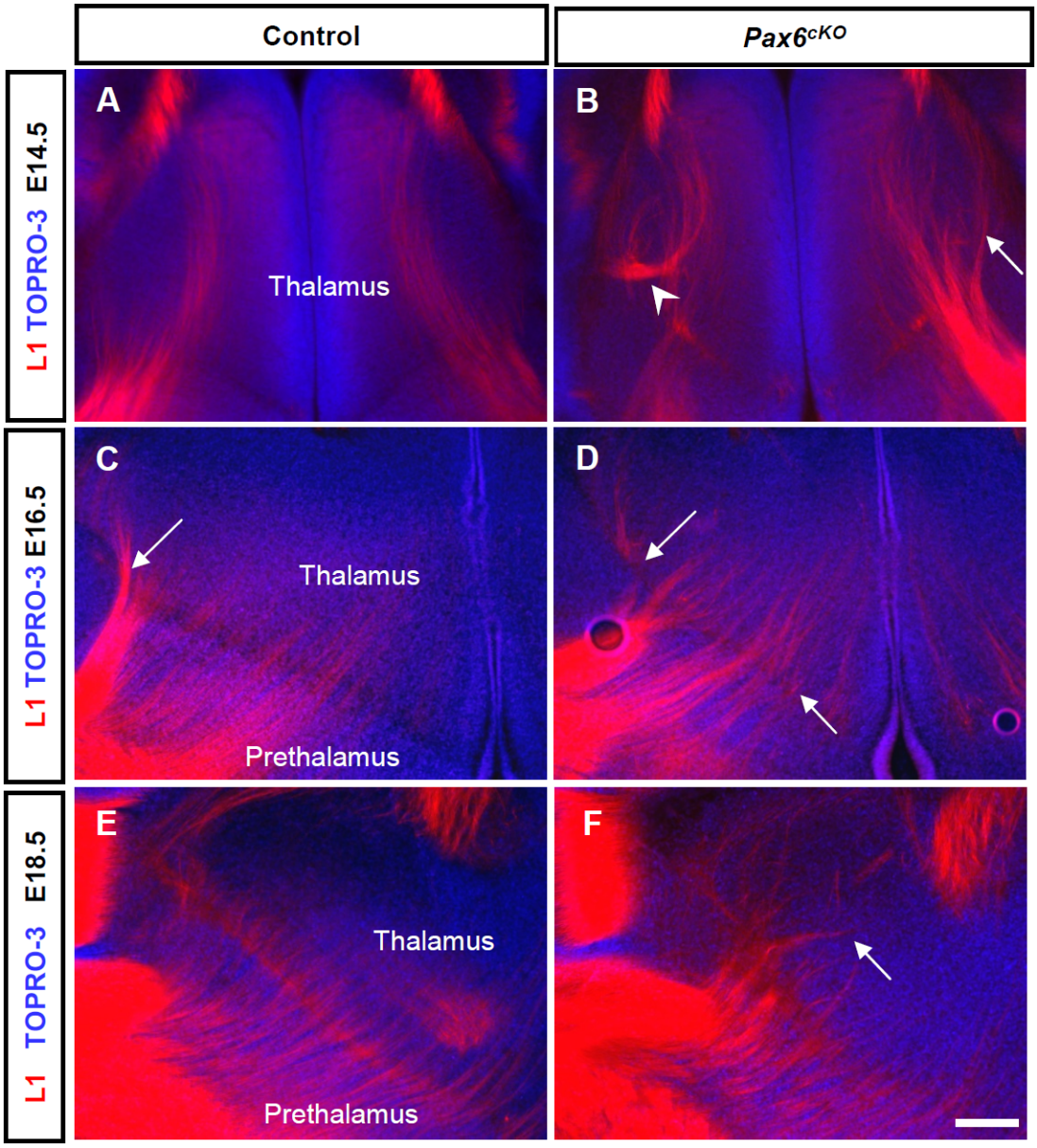


Figure 5. **Immunohistochemistry for axonal marker L1 shows thalamocortical axons appear disorganised in the *Pax6*^{cKO} diencephalon**. L1 Immunohistochemistry at E14.5 (A, B), E16.5 (C, D) and E18.5 reveal the thalamocortical tract within the diencephalon. TCAs become disorganised within the thalamus of the *Pax6*^{cKO} displaying a 'frayed' appearance in comparison to control (arrows B, D, F) and form aberrant axon bundles (arrow head B). All images are higher magnification of equivalent images of the same embryos in figure 4. Scale bar: 200µm.

4.2.5 Gross topography of thalamocortical projections is maintained in the *Pax6^{cKO}* mouse

In the *Pax6$^{Sey/Sey}$* mouse TCAs do not reach the cortex, but in the *Pax6^{cKO}* TCAs do cross the PSPB and reach the cortex, as has been shown above. As TCAs extend towards the cortex, the topography of the projections from the various nuclei of the thalamus is maintained. TCAs that extend from the ventrolateral nucleus project to the motor cortex within the caudal part of the cortex. TCAs from the ventrobasal complex (VB) and the posteriormedial complex (PoM) project to the somatosensory cortex, caudal to the motor cortex. TCAs from the most lateral part of the thalamus, the dorsal lateral geniculate nucleus (dLGN) project to the visual cortex at the caudal most part of the cortex. To determine whether this topographic organisation of the thalamocortical tract is affected in the *Pax6^{cKO}* two-colour tract tracing was used. By E18.5 TCAs have reached their target regions within the cortex. At this stage DiI (red) was injected into the primary somatosensory cortex (S1) and DiA (green) was injected into the primary visual cortex (V1) (Fig. 6C). In this way retrograde DiI and DiA diffusion labels distinct populations of TCAs projecting to the cortex and the cell bodies from which they originate.

In the control DiI placement at S1 in the cortex specifically causes the labelling of VB and PoM in the thalamus. DiA placement at V1 in the cortex labels cell bodies within the dLGN, lateral to the DiI labelling seen in the VB (Fig. 6D, 7A). In the *Pax6^{cKO}* a similar labelling pattern can be observed with DiI at the VB and DiA at the dLGN (Fig, 6E, 7B) (control n = 4, *Pax6^{cKO}* n = 4). This indicates that the gross topography of thalamocortical projections is maintained in the *Pax6^{cKO}* mouse, with TCAs from the VB and PoM projecting correctly to S1 and TCAs from the dLGN projecting correctly to V1. In addition it also appears that the topographic arrangement of corticothalamic projections to the thalamus from the cortex (also labelled during this experiment) are unaffected in *Pax6^{cKO}* embryos when compared to control embryos.

The DiI and DiA labelling pattern however does appear subtly different in the *Pax6^{cKO}* mouse. When looking at the labelled TCAs within the prethalamus it is clear that DiI labelled axons are medial to those labelled with DiA in both genotypes. But these axons, particularly those labelled with DiI, appear abnormally fasciculated in

the prethalamus of the *Pax6*^{cKO} (arrows Fig. 6G, 7E) when compared to the smooth curve of axons seen in the control (Fig. 6F, 7D). This finding is consistent with what has been seen by L1 immunohistochemistry (Fig. 4, 5). The labelling within the thalamus appears more variable in the *Pax6*^{cKO}, with a more patchy appearance than that seen in the control (Fig. 6E, 7B). This patchy staining could be due to the fact that, as the L1 immunohistochemistry has shown, TCAs become disorganised within the thalamus of the *Pax6*^{cKO} (Fig. 5) which may cause fewer TCAs to reach the cortex. Another observation is that although DiA labelled cell bodies were seen within the dLGN of all labelled control and *Pax6*^{cKO} embryos, there appears to be fewer of these labelled cell bodied within the dLGN of *Pax6*^{cKO} embryos compared to control (Fig. 6D, E). This may indicate that there is reduced innervation of S1 by the dLGN in these embryos, although analysis of a larger number of animals will be required to determine if this is the case.

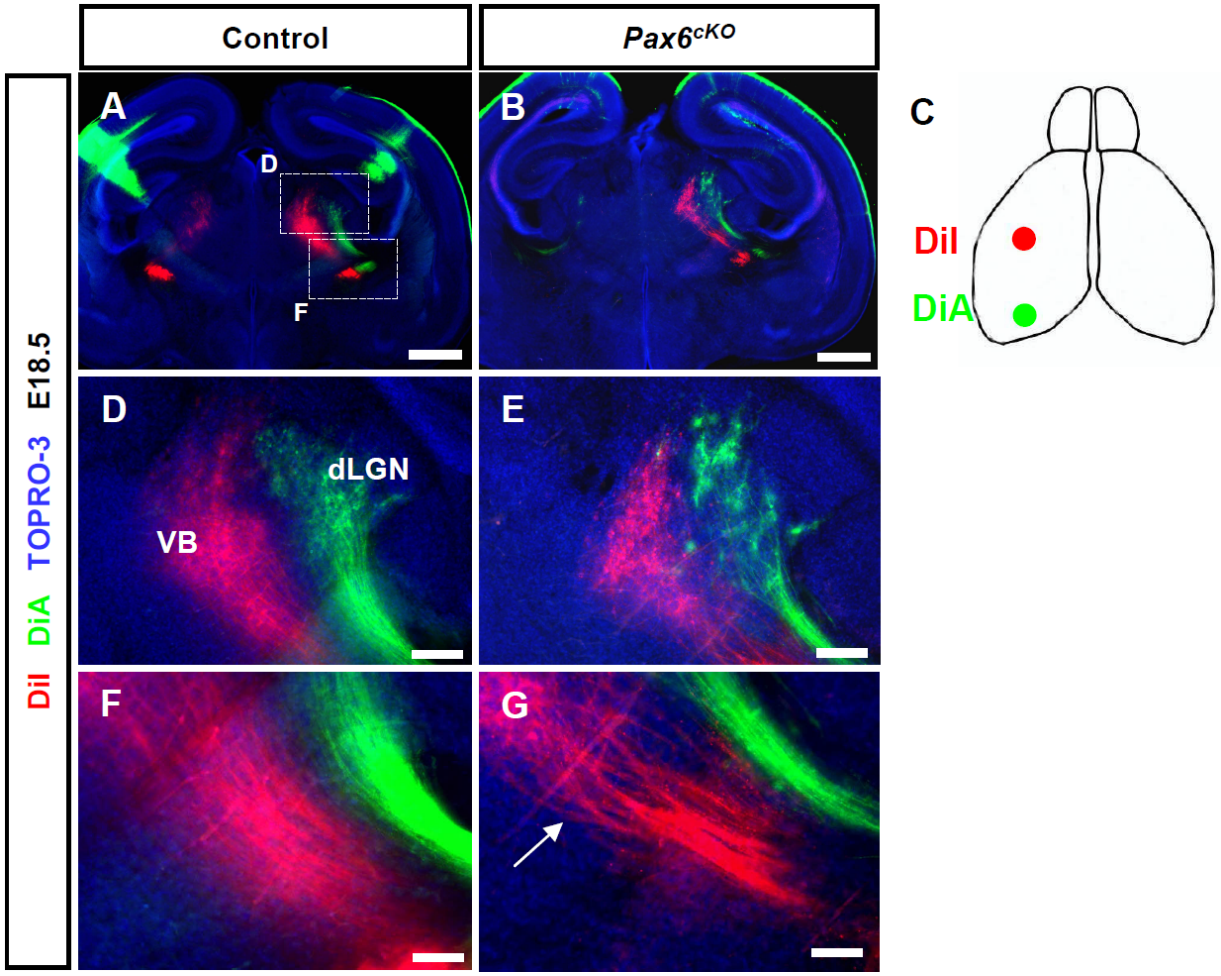


Figure 6. **Gross topographic organisation of the thalamocortical is normal in the *Pax6*^{cKO}. (A, B,)** DiI and DiA was placed into the somatosensory and visual cortex respectively at E18.5**. (C)** Schematic diagram indicating the position of the dye placement sites. **(D, E)** Retrograde DiI and DiA diffusion labels the ventrobasal complex and the dorsal lateral geniculate nucleus respectively in both the control (D) and the *Pax6*^{cKO} (E). **(F, G)** DiI labelled TCAs become abnormally fasciculated in the *Pax6*^{cKO} (arrow, G) when compared to control (F). Scale bars: 500µm in A and B, 200 µm in D-F.

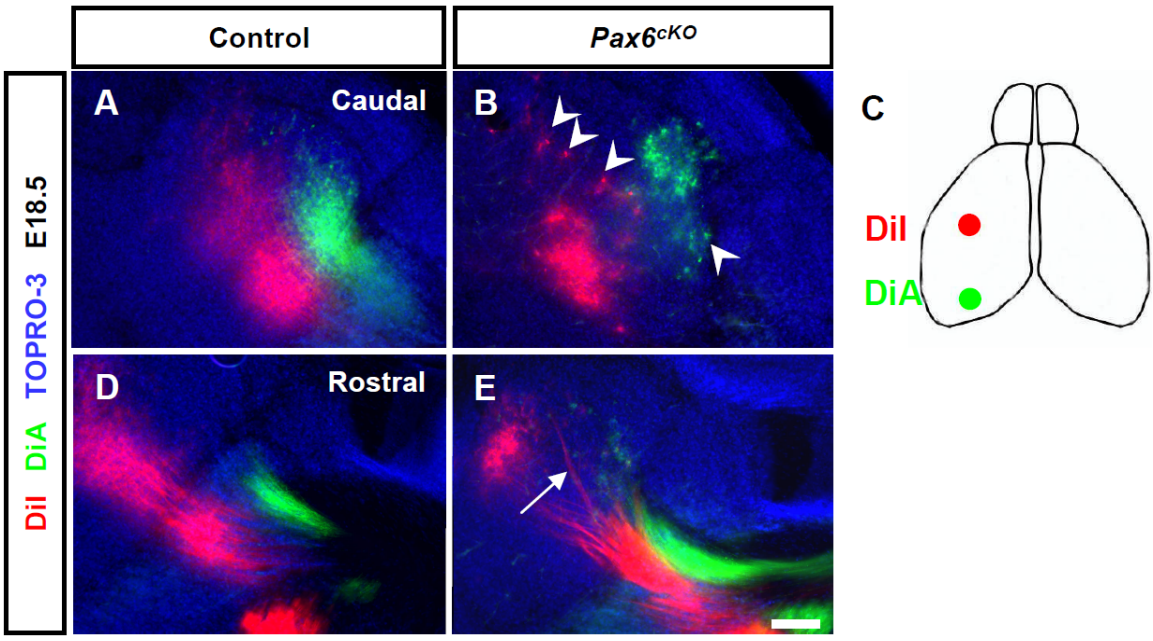


Figure 7. **Labelling of the thalamus following DiI and DiA placement at the cortex is variable in the *Pax6*^{cKO}. (A-E)** Second example of DiI placement at the somatosensory cortex and DiA placement at the visual cortex. **(A, B)** Retrograde DiI and DiA diffusion labels the VB and dLGN respectively in both the control and the *Pax6*^{cKO} as in Fig. 6. Labelling pattern in the *Pax6*^{cKO} appears more patchy with gaps in between clusters of labelled cells in both the VB and the dLGN (arrowheads, B). (D, E) Abnormal fasciculation of the DiI labelled TCAs within the *Pax6*^{cKO} prethalamus (arrow, E) as in Fig. 6. Sale bar 200 µm.

4.2.6 Disrupted pioneer axon development in the *Pax6^{cKO}* diencephalon

One method by which TCAs are thought to be guided through the diencephalon and into the ventral telencephalon is the development of so called 'pioneer axons'. These are formed from transient populations of cells within the internal capsule zone of the ventral telencephalon and reticular nucleus of the prethalamus that extend axons towards the thalamus. These axons then act as a scaffold to guide TCAs through the diencephalon and into the telencephalon (Molnar and Cordery, 1999). Disruption of these pioneer axon tracts is often associated with axon pathfinding defects (Lopez-Bendito et al., 2002; Magnani et al., 2010; Tuttle et al., 1999).

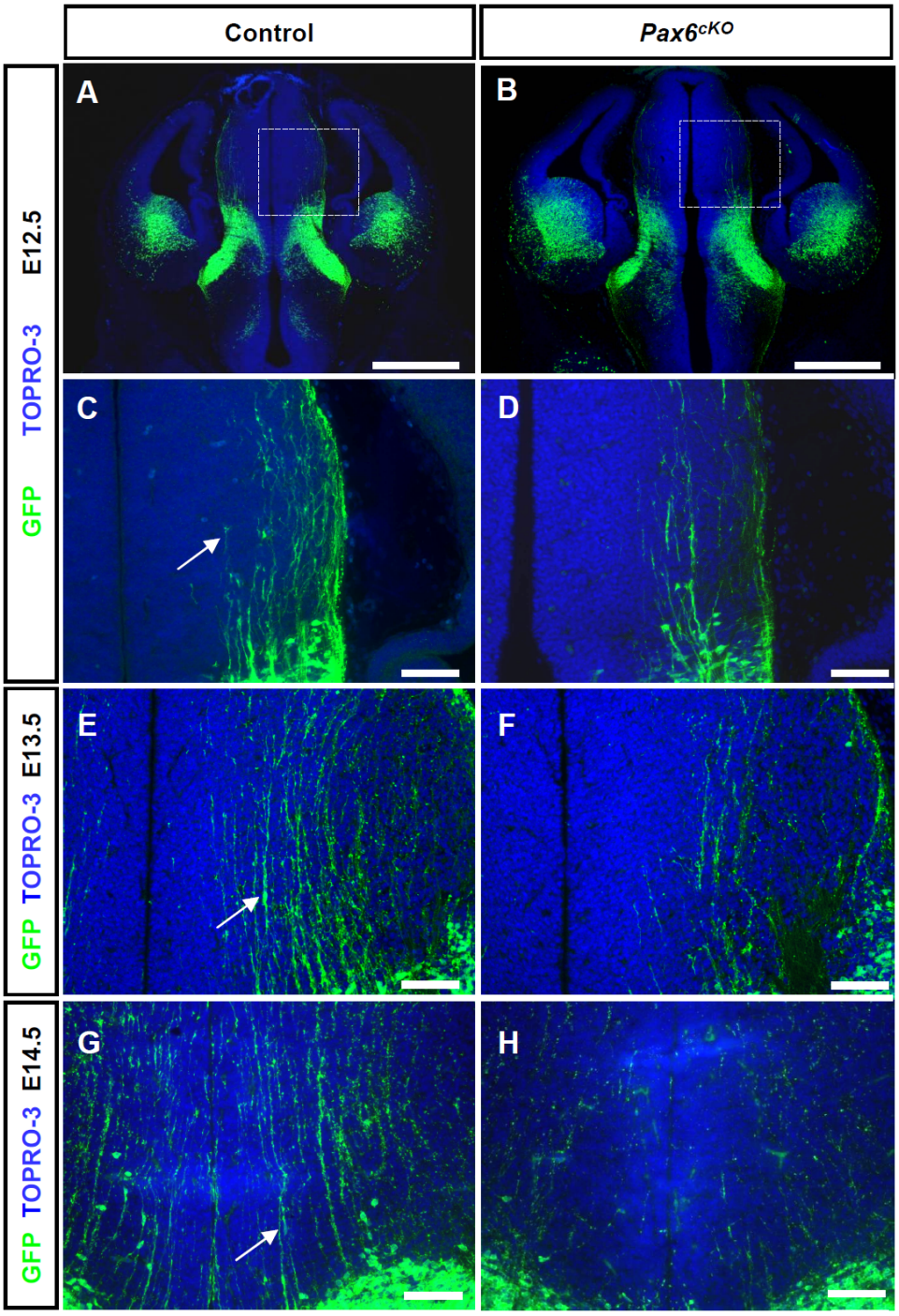
The RCE GFP reporter causes GFP expression in cells where Cre recombinase is active. When this reporter is combined with the *Gsh2* Cre allele, GFP is expressed throughout the prethalamus from around E11.5. In addition to the cells of the prethalamus GFP also labels the pioneer axons which originate from these cells. To examine the development of these pioneer axons immunohistochemistry for GFP was conducted at E12.5, E13.5 and E14.5 in control and *Pax6^{cKO}* embryos. In the control at E12.5 immunohistochemistry for GFP reveals GFP expression at the prethalamus and throughout much of the ventral telencephalon (Fig. 8A). Within the diencephalon pioneer axons can be seen labelled with GFP. These axons extend dorsally from the prethalamus into the thalamus (Fig. 8C). At this stage the pioneer axons are restricted to the lateral half of the thalamus; at the later stage of E13.5 the area covered by these axons extends medially and by E14.5 pioneer axons are found right across the thalamus (Fig. 8E, G). In the *Pax6^{cKO}* the pioneer axons are visible at E12.5 but they appear fewer in number compared to the control (Fig. 8D). At E13.5 and E14.5 there is also a marked reduction in the amount of GFP labelled axons projecting into the thalamus (Fig. 8F, G).

To quantify the reduction in GFP labelled pioneer axons extending from the prethalamus into the thalamus image analysis was conducted using ImageJ. GFP immunohistochemistry was performed on sections which were then imaged, keeping the exposure and the gain constant. Once the images had been obtained, the ImageJ programme was then used to draw a line on a part of the tissue deemed to not contain

any axons. The colour intensity (greyscale value) was then measured across this line; the highest value measured was then used as a background measurement. A second line was then drawn horizontally from the midline of the diencephalon to the lateral edge of the thalamus; this line was placed 100µm dorsal of the prethalamus (Fig. 9A, B). The colour intensity was then measured across this line and where the measurement was above the background value it was deemed that the line was covered by an axon. The proportion of the line covered by axons was then calculated. This method of quantification is a modified version of the method used by (Tian et al., 2008) to quantify axon outgrowth from cultured retinal explants.

Pioneer axon growth was quantified at three stages E12.5, E13.5 and E14.5. For the control the proportion of the line covered by axons increases slightly between E12.5 and E14.5 (Fig. 9C). In the *Pax6*^{cKO} there is a marked reduction in the proportion of the line covered by axons between E12.5 and E14.5, indicating that the number of pioneer axons is reduced in the *Pax6*^{cKO} compared to control. The best fit lines for the control and *Pax6*^{cKO} were analysed by regression analysis and found to be significantly different (P= 0.035, Control n=3, *Pax6*^{cKO} n=3 at each developmental stage). In addition the mean line coverage at E14.5 is significantly reduced in the *Pax6*^{cKO} compared to the control (control 46.88%, SEM ±2.75, n=3. *Pax6*^{cKO} 23.14% SEM ±2.43, n=3. Student's t test P = 0.036). This reduction in the outgrowth of pioneer axons from the *Pax6*^{cKO} prethalamus may go some way to explain why TCAs become disorganised within the thalamus as the guidance the TCAs receive from the pioneer axons is missing in the *Pax6*^{cKO}.

***Figure 8*. GFP reporter combined with the *Gsh2*^{Cre} allele labels pioneer axons extending from the prethalamus into the thalamus. The number of these axons appears reduced in the *Pax6*^{cKO}. (A, B)** Immunohistochemistry for GFP at E12.5 reveals GFP labelled cells within the prethalamus and the ventral telencephalon in both the control and the *Pax6*^{cKO}. **(C, E, H)** In the control GFP labelled pioneer axons can be seen extending from prethalamus into the thalamus at E12.5 (C), E13.5 (E) and E14.5 (G). **(D, F, H)** In the *Pax6*^{cKO} GFP labelled axons are visible but appear fewer in number at each developmental stage. C and D are higher magnification images of the boxed regions in A and B respectively. E-H are the equivalent region to that displayed in C and D at later developmental stages. Scale bars: 500μm A and B, 100μm C-H.


Control
Pax6cKO
A
B
C
D
E
F
G
H
E12.5
TOPRO-3
GFP
E13.5
TOPRO-3
GFP
E14.5
TOPRO-3
GFP

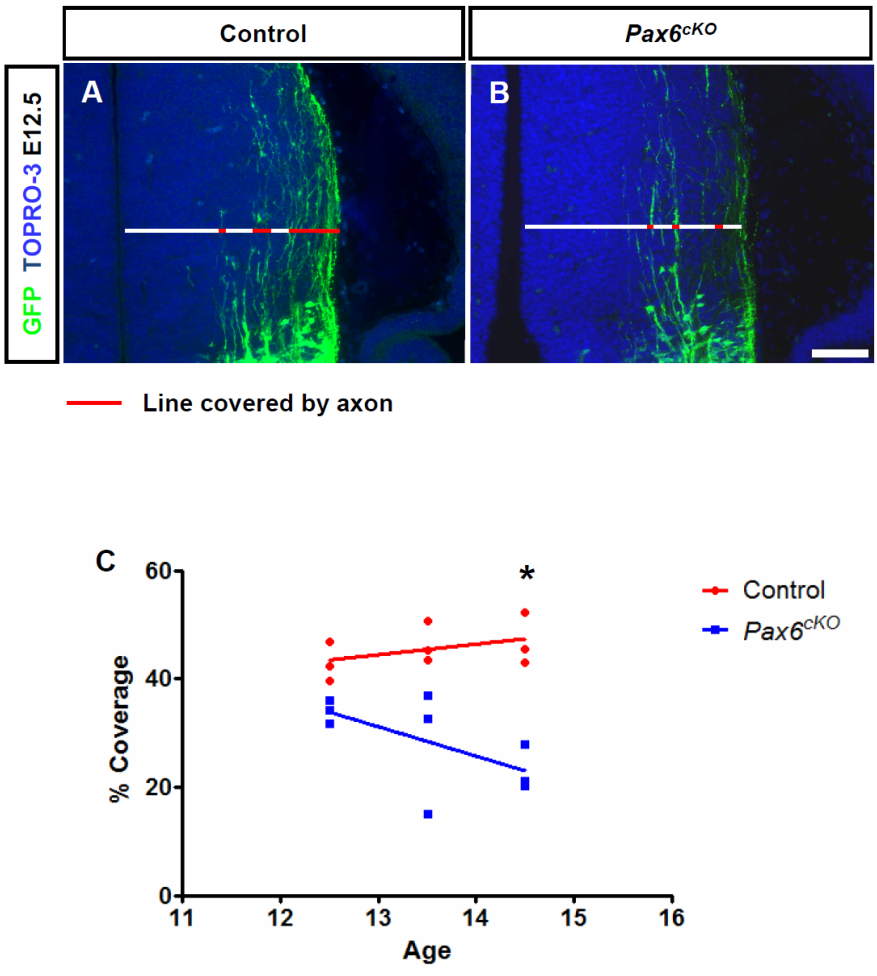


Figure 9. **Quantification of GFP labelled pioneer axons show that pioneer axon outgrowth from the prethalamus is significantly reduced between E12.5 and E14.5 in the *Pax6*^{cKO}. (A, B)** Examples showing quantification method. ImageJ programme was used to measure colour intensity along a horizontal line drawn across the thalamus. Where the intensity value was above background the line was deemed to be covered by axons, the proportion of the line covered by axons was then calculated. **(C)** Quantification of GFP axons in control *Pax6*^{cKO} and between E12.5 and E14.5. Control shows a slight increase in the proportion of the line covered by axons from E12.5 to E14.5. By contrast the *Pax6*^{cKO} shows a marked decrease in line coverage indicating a reduction in pioneer axon outgrowth from the prethalamus. Note: Images were quantified using only green GFP channel, blue TOPRO-3 channel is included in A and B to show tissue morphology. Scale bar: 100μm.

4.2.7 Prethalamic pioneer axons originate from cells that express *Pax6*

In order to determine if the prethalamic pioneer axons originate from *Pax6* expressing cells the DTy54 *Pax6* GFP reporter mouse was used. The DTy54 reporter mouse expresses GFP under the control of human *Pax6* regulatory elements; this causes the expression of GFP in cells where *Pax6* is expressed (Tyas et al., 2006). This mouse was crossed with the $Gsh2^{Cre}$ and $Pax6^{Flox/Flox}$ mice in the absence of the RCE GFP reporter allele to create $Pax6^{cKO}$ mice that express GFP in cells that express *Pax6* or cells where the *Pax6* promoter is active (rather than cells that express Cre recombinase). This means the $Pax6^{cKO}$ embryos are $Gsh2^{Cre+/-}$, $Pax6^{Flox/Flox}$, DTy54 and control embryos are $Gsh2^{Cre}$, $Pax6^{+/+}$, DTy54. Due to the cytoplasmic localisation of the GFP from the DTy54 reporter this approach allows the labelling of axons in a similar fashion to that seen using the RCE GFP reporter.

In both the control and the $Pax6^{cKO}$ at E14.5 GFP expression can be seen in Pax6 expressing regions such as the cortex and the prethalamus (Fig. 10A, B). In the thalamus of the control, GFP can be seen labelling axons extending from the prethalamus. These axons closely resemble those that have been labelled using the RCE GFP reporter. This indicates that the pioneer axons described in Fig. 8 originate from *Pax6* expressing cells. In the $Pax6^{cKO}$ these axons appear fewer in number compared to the control; this is consistent with the observations in figures 8 and 9. This shows that the loss of *Pax6* in prethalamic cells causes a reduction in pioneer axon outgrowth and suggests that *Pax6* plays a cell autonomous role in the outgrowth or maintenance of pioneer axons required for the guidance of TCAs.

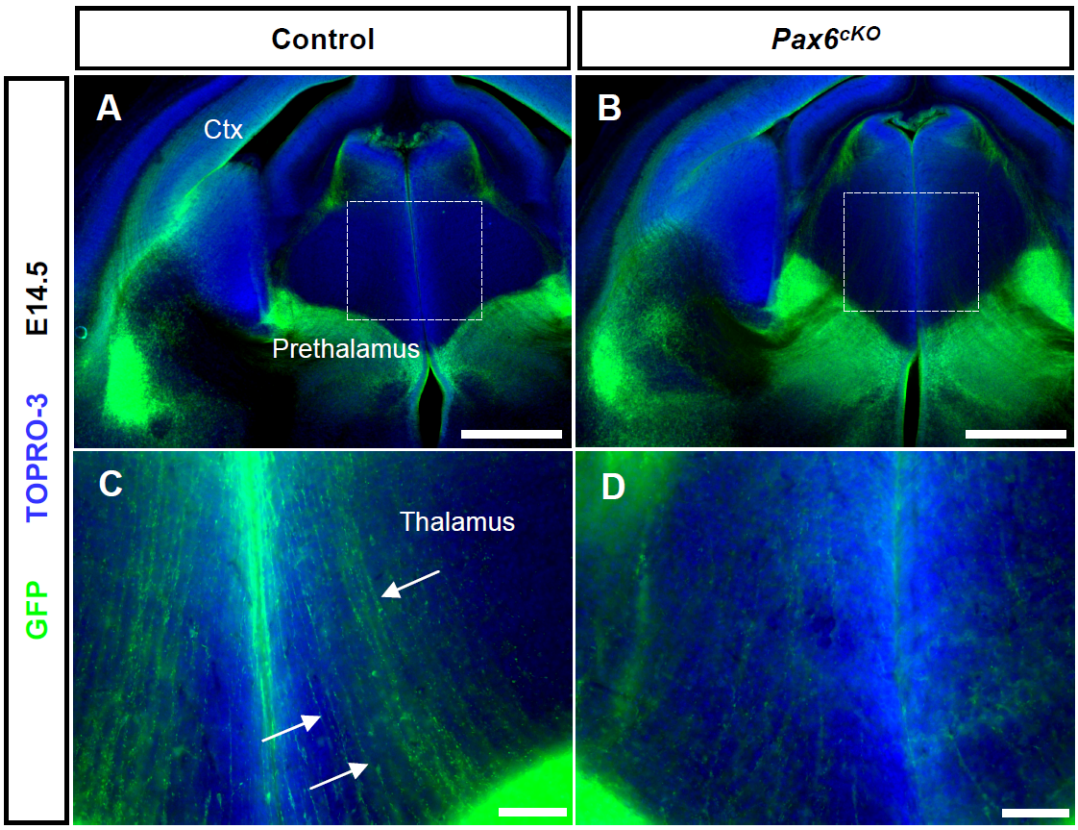


Figure 10. **Pioneer axons extending from the prethalamus originate from *Pax6* expressing, cells.** Using the DTy54 *Pax6* reporter allele the *Pax6* expressing cells were transgenically labelled with GFP in both the control and the $Pax6^{cKO}$. **(A, C)** Immunohistochemistry for GFP in the control at E14.5 reveals cells which express *Pax6*. GFP expression can be seen in *Pax6* expressing regions including the cortex and the prethalamus (A). GFP expression can also be seen in the pioneer axons that extend from the prethalamus (C). **(B, D)** In the $Pax6^{cKO}$ GFP expression is present at the cortex and the prethalamus (B). The prethalamic pioneer axons are absent in the $Pax6^{cKO}$. C and D are higher magnification images of the boxed regions in A and B respectively. Scale bars 500µm in A and B, 100µm in C and D.

4.2.8 No evidence for a change in the rate of apoptotic cell death within the prethalamus of the *Pax6^{cKO}*.

One possible explanation for the reduction in the pioneer axon outgrowth from the prethalamus is that the cells from which these axons originate are dying due to the loss of *Pax6*. To asses this immunohistochemistry for cleaved *Caspase-3* was performed at E12.5, E13.5 and E14.5. *Caspase3* undergoes proteolytic cleavage when activated during apoptosis and thus the cleaved form of *Caspase3* is a marker of cells undergoing apoptosis. Between E12.5 and E14.5 very few cells within the prethalamus of control embryos express cleaved *Caspase3* (arrows, Fig.11A, C, E). This is also the case for *Pax6^{cKO}* embryos (Fig .11B, D, F). The number of cleaved *Caspase3* expressing cells found in the control and prethalamus was quantified and no significant difference was found between the two genotypes (Student's t test p= 0.58, control n=3, *Pax6^{cKO}* n=3) (Fig. 11G). This result suggests that there is no increase in apoptotic cell death within the prethalamus of the *Pax6^{cKO}* and that the loss of prethalamic pioneer axons in this mouse is not due to death of the cells which extend pioneer axons. It should be remembered though that the Caspase-3 antibody will only label cells during apoptosis, while cells which have already undergone death and been cleared will not be labelled. This means that Caspase3 immunohistochemistry will only give a 'snapshot' of apoptosis occurring at the precise time the embryo was sacrificed, and any subtle change in the rate of cell death may not be detected. Nonetheless this result confirms that there is no large change in the rate of cell death within the prethalamus of these embryos.

4.2.9 Patterning of the prethalamus is unchanged in the *Pax6^{cKO}*.

Pax6 is known to play an important role in the patterning of the forebrain, particularly the dorsoventral patterning of the telencephalon (Manuel and Price, 2005). The *Pax6$^{Sey/Sey}$* telencephalon displays severe patterning defects in particular a ventralisation of the neocortex (Stoykova et al., 2000). The diencephalon also experiences patterning abnormalities for example the expression domain of *Sonic hedgehog* (*Shh*), a marker for the zona limitans intrathalamica (ZLI) is expanded in the *Pax6$^{Sey/Sey}$* embryo (Grindley et al., 1997). The changes seen in the molecular regionalisation of the prethalamus may contribute to the TCA pathfinding defects

seen in the *Pax6*$^{Sey/Sey}$ diencephalon (Pratt et al., 2000b). Any alteration in the patterning of the prethalamus may have a profound impact on the development of the prethalamic pioneer axons, and may explain the reduction in pioneer axon outgrowth seen in the *Pax6*^{cKO} embryo. In order to investigate the patterning of the prethalamus in the *Pax6*^{cKO} the expression of a number of diencephalic marker genes was analysed by *in situ* hybridisation and immunohistochemistry at E13.5. *Ngn2* is expressed throughout the thalamus at E13.5 (Fode et al., 2000), in both the control and *Pax6*^{cKO} embryos *Ngn2* staining forms a sharp boundary where the thalamus meets the prethalamus (Fig 12A, B). *Shh* is a marker for the ZLI and its expression at this position is important for the development of both the thalamus and the prethalamus (Figdor and Stern, 1993; Kiecker and Lumsden, 2004; Vue et al., 2009). A small notch of *Shh* expression can be seen close to the midline of the diencephalon in both control and *Pax6*^{cKO} embryos (Fig 12B, C) indicating that the ZLI is intact in *Pax6*^{cKO} embryos. Transcription factors *Islet1* and *Lim1/2* are expressed throughout the prethalamus (Nakagawa and O'Leary, 2001; Wang and Liu, 2001). In both control and *Pax6*^{cKO} embryos *Lim1/2* staining can be seen in both postmitotic neurons and progenitor cells of the ventricular zone, while *Islet1* staining is only found in postmitotic cells (Fig. 12E-H). *Mash1* is expressed in the progenitor cells of the ventricular zone of the prethalamus of the control (Guillemot and Joyner, 1993; Porteus et al., 1994) and this is unchanged in the *Pax6*^{cKO} embryos, in this example the fourth ventricle is enlarged compared to control (asterisk, Fig. 12I) as is the case in approximately 1/3 of cases (Fig 12I, J). *Nkx2.2* is expressed at the ventricular zone of the prethalamus and within a strip of postmitotic cells along the dorsal edge of the prethalamus, expression can also be seen at the region which will become the ventral lateral geniculate nucleus (Kitamura et al., 1997) (Fig. 12K). This expression pattern is maintained in *Pax6*^{cKO} embryos (Fig. 12L).

The cell adhesion molecule *R-cadherin* is expressed throughout the prethalamus (Fig. 12M). Previous studies have identified that *R-cadherin* is *Pax6* regulated, and that its expression at the prethalamus is required for the growth of pioneer axons which guide the TPOC (Andrews and Mastick, 2003; Nural and Mastick, 2004). It is possible that a change in *R-cadherin* expression may cause the reduction in pioneer axon outgrowth seen in the *Pax6*^{cKO} embryo. *R-cadherin*

expression within the *Pax6*^{cKO} prethalamus appears unchanged (Fig. 12N) which suggests that this is not the case. It is possible though that any change in the level of *R-cadherin* expression may be too subtle to detect using immunohistochemistry.

From this gene expression analysis we can conclude that there is no major change in the patterning of the prethalamus in the *Pax6*^{cKO}. This suggests that the cell populations which will extend pioneer axons into the thalamus are likely to be present in the *Pax6*^{cKO}.

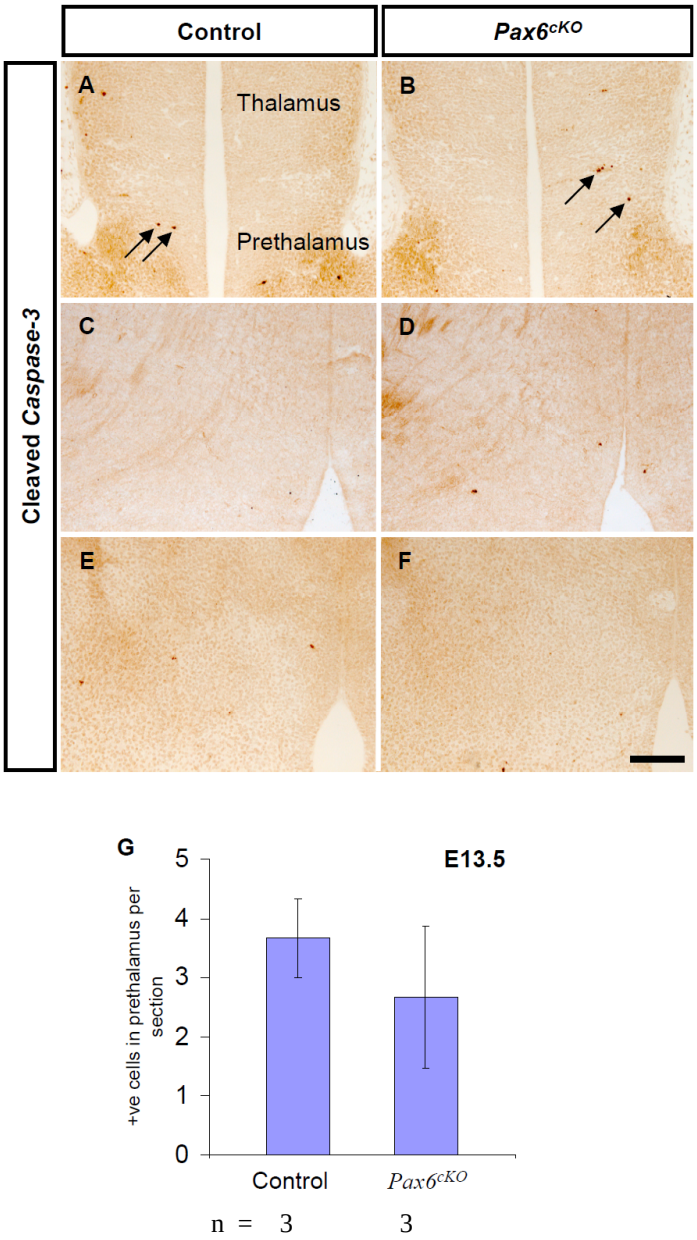
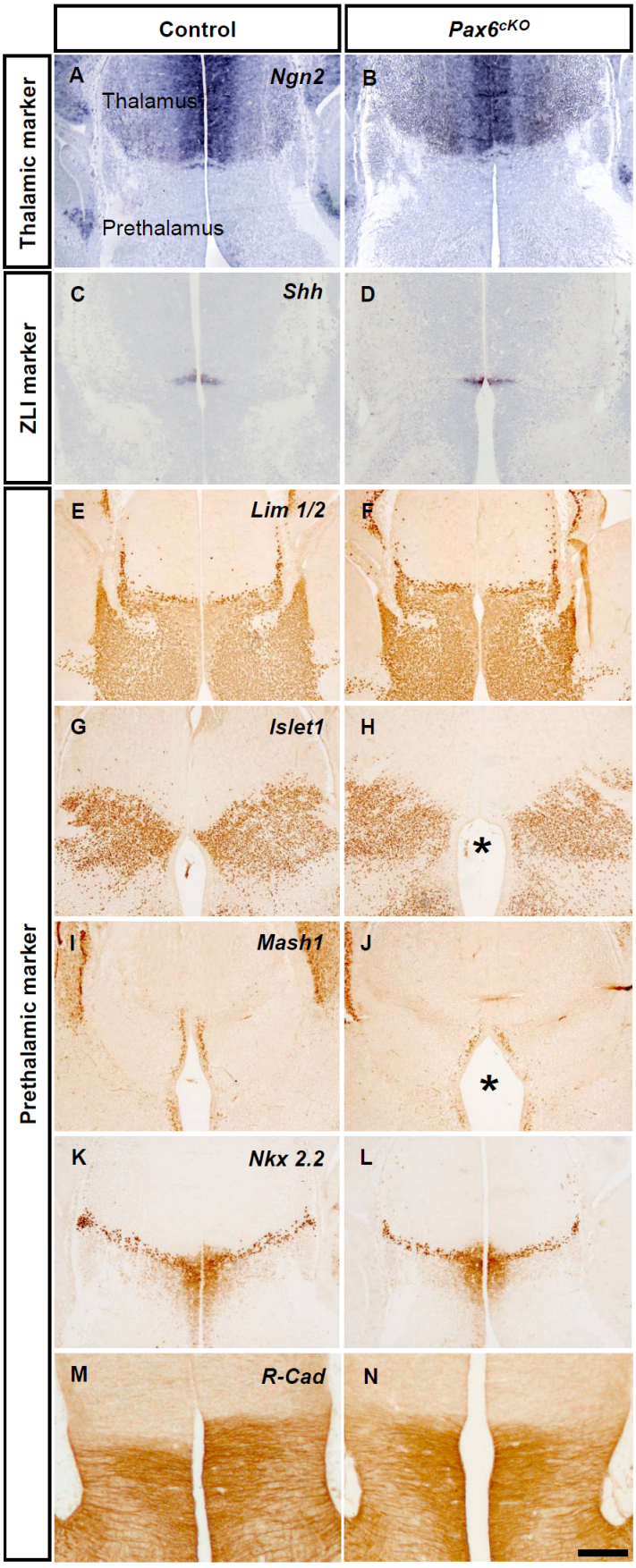


Fig 11. **The rate of cell death appears unchanged in the *Pax6*^{cKO} prethalamus.** **(A-F)** Immunohistochemistry for cleaved *Caspase-3* at E12.5 (A, B), E13.5 (C, D) and E14.5 (E, F) was used to assess the rate of cell death in the prethalamus of the control and the *Pax6*^{cKO}. A small number of cells within the prethalamus express cleaved *Caspase-3* in both the control and the *Pax6*^{cKO}. **(G)** Quantification of the number of cleaved *Caspase-3* expressing cells within the prethalamus at E13.5 shows no significant difference between control and *Pax6*^{cKO}. Scale bar 100μm.

Figure 12. **The patterning of the prethalamus is normal in the *Pax6*^{cKO}**. *In situ* hybridisation (A-D) and immunohistochemistry (E-L) has been used to analyse the molecular patterning of the prethalamus at E13.5. **(A, B)** Expression of thalamic marker *Ngn2* is found throughout the thalamus in both the control (A) and the *Pax6*^{cKO} (B). **(C, D)** *Sonic Hedgehog* is a marker for the Zona Limitans Intrathalamica (ZLI) which forms the boundary between the thalamus and the prethalamus, *Shh* expression is normal in the *Pax6*^{cKO} (D).**(E-L)** Expression of prethalamic markers *Lim1/2* (E, F), *Islet1* (G, H), *Mash1* (I, J) and *Nkx2.2* is unchanged in the *Pax6*^{cKO}. Scale bar 200μm.


Control
Pax6cKO
Thalamic marker
ZLI marker
Prethalamic marker
A
Ngn2
Thalamus
Prethalamus
B
C
Shh
D
E
Lim 1/2
F
G
Islet1
H
*
I
Mash1
J
*
K
Nkx 2.2
L
M
R-Cad
N

4.2.10 Patterning is disrupted close to the pallial-subpallial boundary of the *Pax6^{cKO}*.

In addition to the prethalamus, *Pax6* expression is also deleted within the LGE of the ventral telencephalon, close to the PSPB (Fig. 1). The patterning of the ventral telencephalon was examined at E13.5 using immunohistochemistry and *in situ* hybridisation. In the control *Dbx1* is expressed by a small patch of cells at the pallial side of the PSPB (Medina et al., 2004)(Fig. 13A) but in the *Pax6^{cKO} Dbx1* expression at this position is almost entirely lost (arrow, Fig. 13B). *Ngn2* is also expressed on the pallial side of the PSPB and throughout the ventricular zone of the cortex (Fode et al., 2000)(Fig. 13C). In the *Pax6^{cKO} Ngn2* expression is reduced within the ventral telencephalon, with the edge of the expression domain retracted dorsally (Fig. 13D). This indicates that the patterning of the PSPB region is disrupted in the *Pax6^{cKO}* although this disruption is much less profound than the global patterning abnormalities seen in the *Pax6$^{Sey/Sey}$* mouse (published in Cocas et al 2011).

Islet1 is a marker for postmitotic cells of the LGE (Wang and Liu, 2001), it is also expressed by cells of the axon permissive 'corridor' within the MGE. These corridor cells migrate tangentially from the MGE and have been shown to be required for the correct guidance of TCAs through the ventral telencephalon (Lopez-bendito et al, 2006). In control embryos *Islet1* expression can be seen clearly throughout the LGE and at the narrow corridor within the MGE (Fig. 13E). In the *Pax6^{cKO}* expression is maintained at the LGE and the corridor cells can clearly be seen within the LGE (Fig. 13F). This shows that the *Islet1* expressing corridor cells migrate to the MGE normally in the *Pax6^{cKO}*. This may explain why TCAs navigate normally through the ventral telencephalon.

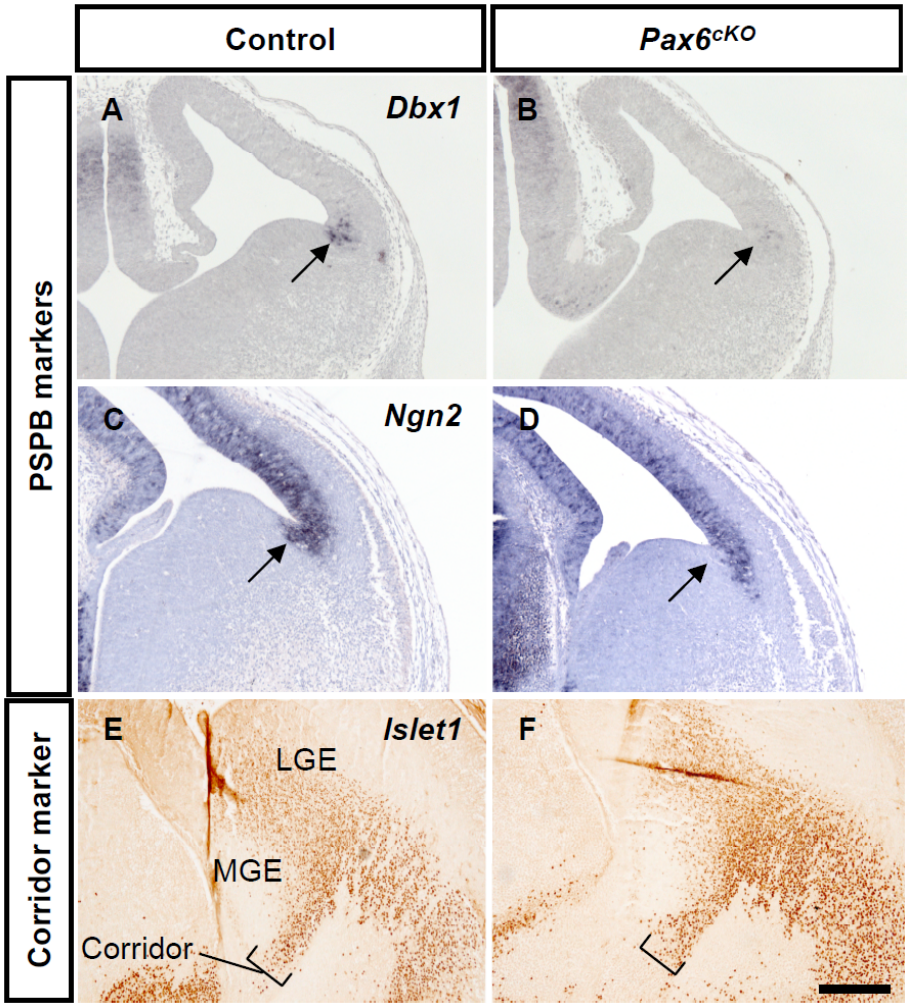


Figure 13. **Patterning defects can be observed at the Pallial-subpallial boundary of the *Pax6*^{cKO} .** **(A-D)** Transcription factors *Dbx1* and *Ngn2* are markers of the pallial side of the PSPB. *In situ* hybridisation shows *Dbx1* expression in the ventral pallium in the control (A) in the *Pax6*^{cKO} *Dbx1* expression is markedly reduced (B). In the control *Ngn2* expression can be seen throughout the ventricular zone of the pallium up to the PSPB (arrow, C). In the *Pax6*^{cKO} *Ngn2* expression is reduced in the ventral pallium (arrow, D). **(E, F)** *Islet1* is a marker for the LGE and the axon permissive corridor within the MGE. *Islet1* expression is unchanged in the *Pax6*^{cKO} (F). Scale bar 200µm.

4.3 Discussion

4.3.1 Summary

In this chapter we have seen that when *Pax6* expression is specifically reduced within the murine prethalamus (*Pax6*^{cKO} embryos) TCA guidance is disrupted within the diencephalon. This result demonstrates that *Pax6* gene expression is required within these cells for the correct formation of the thalamocortical tract.

Use of the *Gsh2Cre* allele (Kessaris et al., 2006) in combination with the lox stop (RCE) GFP reporter allele (Sousa et al., 2009) in this chapter has provided a novel way of labeling pioneer axons which originate from cells within the prethalamus and extend into the thalamus. These axons are proposed to guide TCAs from the thalamus into the prethalamus (Molnar et al., 1998a). When *Pax6* expression is reduced within the prethalamus the development of this pioneer axon tract is disrupted which shows that *Pax6* expression is required for the formation of this tract. Analysis of molecular markers for prethalamic cells has shown that patterning within the prethalamus of *Pax6*^{cKO} embryos appears normal, which suggests that the primary cause of the TCA guidance defects observed in these embryos is the disruption of pioneer axons. The association between disruption prethalamic pioneer axons and TCA guidance errors within the diencephalon observed in *Pax6*^{cKO} embryos provides further evidence that these axons are functionally required for the normal thalamocortical development. The precise molecular mechanism causing the abnormal pioneer axon tract formation in *Pax6*^{cKO} embryos remains unclear.

4.3.2 Thalamocortical axons reach the cortex in the *Pax6*^{cKO} embryo but become disorganised within the diencephalon

The axon tract tracing and L1 immunohistochemistry experiments described in this chapter demonstrate that TCAs reach the cortex in *Pax6*^{cKO} embryos. This is despite the reduction in *Pax6* expression seen in the prethalamus and the ventral telencephalon of these mice. This is in contrast to the *Pax6*$^{Sey/Sey}$ where TCAs fail to cross into the telencephalon and do not reach the cortex (Pratt et al., 2002). In the

Pax6^{cKO}, TCAs extend through the ventral telencephalon and cross the PSPB normally. This is despite patterning abnormalities seen at the PSPB. In two other *Pax6* conditional knockouts TCAs also reach the cortex. In the cortex specific conditional knockout created using the *Emx1 Cre,* TCAs reach the cortex normally (Pinon et al., 2008). In the ventral telencephalic conditional knockout created using the *Six3 Cre* the majority of TCAs cross the PSPB to reach the cortex but unlike the *Emx1 Cre* conditional mutants a small number of TCAs project aberrantly at the DTB and at the internal capsule (Simpson et al., 2009). *Pax6* expression is lost at the amygdaloid region of the *Pax6*^{cKO} in a similar position to *Pax6* deletion seen using the *Six3 Cre.* TCA guidance defects at the DTB and within the ventral telencephalon are not observed in the *Pax6*^{cKO}, but they are seen in the *Six3 Cre* conditional mutant. An explanation for this comes from the fact that the Islet1 expressing corridor that guides axons through the ventral telencephalon forms normally in the *Pax6*^{cKO} unlike the *Six3 Cre* conditional knockout where it is abnormally widened. This difference is likely to be caused by the two Cre alleles driving the deletion of *Pax6* in different populations of cells or at a different developmental time point.

Although TCAs are able to reach the cortex of the *Pax6*^{cKO} mouse, L1 immunohistochemistry shows that TCAs become disorganized within the thalamus and form abnormal bundles that head laterally instead of ventrally. In addition TCAs become abnormally fasciculated within the prethalamus. This disorganisation of TCAs within the thalamus is reminiscent of the behaviour of axons in the *Pax6*$^{Sey/Sey}$ mouse, although this disorganisation is much less severe in *Pax6*^{cKO} embryos. Despite TCAs becoming disorganised within the thalamus the gross topographic arrangement of the TCAs as they project to the cortex is maintained; this is also the case in the cortical conditional knockout (Pinon et al., 2008). The TCA pathfinding errors observed within the thalamus occur despite the fact that *Pax6* expression is unaffected within this part of the *Pax6*^{cKO} brain. This suggests that the prethalamus influences the guidance of TCAs within the thalamus and that *Pax6* is involved in this process.

4.3.3 *Pax6* expression is required at the prethalamus for the formation of the pioneer axon tract that guides TCAs through the diencephalon.

There are two different populations of cells that extend pioneer axons responsible for guiding TCAs through the diencephalon and into the telencephalon. One population extends from cells found at the internal capsule zone within the ventral telencephalon to the thalamus. The second group of cells is found within the reticular nucleus of the prethalamus and also extends axons dorsally into the thalamus (Molnar and Cordery, 1999). These two axon tracts are thought to act as a scaffold to provide guidance to growing TCAs (Molnar et al., 1998a). The latter group of axons which originate from the prethalamus are labeled using the RCE GFP reported in combination with the *Gsh2Cre*. This is a novel technique for labeling these axons which have only previously been identified by retrograde DiI diffusion following DiI placement within the thalamus (Mitrofanis and Baker, 1993). The pioneer axons can be observed from E12.5 and degenerate after E14.5. Thus they are in the correct position at the correct time to guide TCAs leaving the thalamus. Use of the DTy54 *Pax6* GFP reporter mouse has shown that these axons originate from *Pax6* expressing cells. In the $Pax6^{cKO}$ embryo the number of pioneer axons is reduced when compared to WT and the distribution of these axons appears altered from E12.5 onwards. The reduction in the number of pioneer axons is progressive and becomes more pronounced from E12.5 to E14.5. By E14.5 there is a significant reduction in the amount of prethalamic pioneer axons present in the thalamus of $Pax6^{cKO}$ embryos compared to control. This shows that normal *Pax6* expression is required at the prethalamus for the formation of this pioneer axon tract.

This reduction in pioneer axons provides an explanation as to why TCAs become disorganised in thalamus of $Pax6^{cKO}$ embryos. With fewer pioneer axons there will be less guidance for TCAs within the diencephalon leading to axon pathfinding errors such as those seen in the thalamus of this mutant mouse. This also explains how the loss of *Pax6* specifically within the prethalamus affects TCA guidance within the thalamus where *Pax6* expression is normal. This finding provides further evidence that pioneer axons extending from the prethalamic reticular nucleus are required for the correct formation of the thalamocortical tract. In other

mutant mouse models TCA pathfinding defects have been associated with the loss of pioneer axon populations (Lopez-Bendito et al., 2002; Tuttle et al., 1999), but in these instances the embryos have experienced severe morphological or molecular patterning defects which may have been the primary cause of aberrant TCA guidance. *Pax6*^{cKO} Embryos do not experience these morphological or patterning abnormalities so it is most likely that the loss of guidance from pioneer axons is the primary reason for the TCA pathfinding defects that have been observed.

This progressive loss of pioneer axons within the thalamus is due to the loss of *Pax6* expression within cells of the prethalamus, but the precise mechanism causing this to happen is unclear. It may be that the cells which extend these axons die in the *Pax6*^{cKO} causing the loss of pioneers. This hypothesis was tested by examining the rate of apoptosis in the *Pax6*^{cKO} and control. It was found that the rate of apoptotic cell death was unchanged in the *Pax6*^{cKO} prethalamus indicating that cell death was not responsible for this reduction in pioneer axons. *Pax6* is known to play an important role in the patterning of the developing diencephalon (Grindley et al., 1997). Altered molecular patterning within the prethalamus may cause cells which extend pioneer axons to be misspecified. However this is probably not the case as an examination of a number of diencephalic molecular markers has shown that there is no significant change in the patterning of the diencephalon of *Pax6*^{cKO} embryos. The fact that no patterning abnormalities are identified at the prethalamus suggests that the molecular character of the prethalamus is not radically altered in *Pax6*^{cKO} embryos as is the case for *Pax6*$^{Sey/Sey}$ embryos. This leads us to the conclusion that pioneer axon defects are the primary cause of TCA pathfinding errors in the thalamus rather than major molecular changes within the prethalamus.

In control embryos the proportion of the thalamus covered by pioneer axons (as defined by the quantification method used in this study) increases slightly between E12.5 and E14.5. As the width of the thalamus also increases over this time, new pioneer axons must be growing into the thalamus for this to occur. The reduction in the amount of axons by E14.5 in the *Pax6*^{cKO} may be due to the size of the thalamus increasing while fewer new axons are extending from the prethalamus than in the control, resulting in a ‘dilution’ of the existing axons. This would suggest that prethalamic cells lacking *Pax6* either cannot extend these pioneer axons or that they

cannot cross the ZLI to reach the thalamus. To examine the ability of these cells to extend pioneer axons, explants of control and *Pax6*^{cKO} prethalamus could be cultured and the axon outgrowth determined for each genotype. Reduced axon outgrowth in the *Pax6*^{cKO} would indicate that these cells have reduced ability to extend pioneer axons and that *Pax6* is involved in the process of extending the axon from the cell. No change in outgrowth would suggest that pioneer axons grow from the *Pax6*^{cKO} prethalamus but that they cannot reach the thalamus, suggesting a role for *Pax6* in the guidance of the growing axon.

Pax6 has been previously shown to be involved in pioneer axon tract formation. The tract of the postoptic commissure (TPOC) originates from cells close to the optic stalk and extends through the prethalamus and thalamus to reach the epithalamus/habenula. TPOC axons are thought to be guided by a series of pioneer axon populations which lie along the route of TPOC and develop between E9.5 and E11.5 (Mastick and Easter, 1996). TPOC is severely malformed in the *Pax6*$^{Sey/Sey}$ mouse (Mastick et al., 1997); the axons which pioneer this tract do not form correctly, suggesting that *Pax6* is required for the correct guidance of these axons. TPOC pioneer axons cross from the prethalamus into the thalamus crossing the ZLI, but in *Pax6*$^{Sey/Sey}$ embryos the axons do not cross this boundary and instead extend along the dorsal edge of the prethalamus (Nural and Mastick, 2004). This inability of TPOC pioneers to reach the thalamus is similar to the situation in *Pax6*^{cKO} embryos where prethalamic pioneer axons do not reach the thalamus. Expression of *Pax6* regulated cell adhesion molecule *R-cadherin* is lost from the prethalamus and from TPOC pioneer axons in the *Pax6*$^{Sey/Sey}$ mouse (Stoykova et al., 1997). Rescue experiments have shown when *R-cadherin* is reintroduced into the forebrain of *Pax6*$^{Sey/Sey}$ embryos using electroporation TPOC pioneer axons can cross the ZLI (Andrews and Mastick, 2003). This demonstrates that *R-cadherin* plays a role in pioneer axon guidance and may be particularly important for allowing pioneers to cross the ZLI. It is possible that altered *R-cadherin* expression could account for the pioneer axon guidance defects seen in *Pax6*^{cKO} embryos, but at E13.5 the expression pattern of *R-cadherin* appears unchanged in these mice. Further investigation of *R-cadherin* expression within the prethalamus would be needed to conclude whether it plays a role in the guidance of prethalamic pioneer axons.

Evidence from previous studies and work in chapter three has shown that *Pax6* can influence the expression of molecular guidance molecules (Jones et al., 2002). One possible cause of the disruption in pioneer tract formation is that expression of guidance cues is altered in pioneer cells within the prethalamus of $Pax6^{cKO}$ embryos. In particular altered expression of guidance receptors such as *Robos* or *Neuropilins* would affect the ability of pioneer axons to respond to guidance cues present throughout the rest of the diencephalon where *Pax6* expression is unaffected. Immunohistochemistry and *in-situ* hybridization could be used to examine the expression patterns of guidance receptors. In order to look at changes in the level of gene expression, fluorescence-activated cell sorting (FACS) could be used to isolate GFP expressing cells from the prethalamus and qRT-PCR performed to quantify the level of gene expression. Genes of particular interest would be *Netrin1* receptors *DCC* and *Unc5a*, receptors for *Semaphorins* such *Nrp1*and *Slit* receptors *Robo1* and 2. Cell adhesion molecule expression could also be evaluated particularly those molecules thought to be regulated by *Pax6* such as *R-cadherin* (described above) and *OB-cadherin*.

This technique for labeling the prethalamic pioneer axons provides a useful tool to further study this axon tract. The combination of the $Gsh2^{Cre}$ and the RCE GFP reporter alleles with other mutant mice, for example other transcription factor mutants such as the *Emx2* knockout, would allow for the examination of the role that other regulatory genes play in the development of pioneer axon tracts. Use of this reporter with mice with guidance molecule mutations would also tell us which guidance cues may be vital for the guidance of pioneer axons. Although it is clear that these axons originate from *Gsh2* lineage cells that express *Pax6*, little else is known about the molecular character of these cells. As presumably only a small proportion of prethalamic cells labeled with GFP project axons, further analysis of molecular markers, possibly in conjunction with DiI labeling of pioneers, could allow for a better characterisation of these cells. Leading to a greater understanding their development and the role they play in the guidance of the thalamocortical tract.

Chapter 5: The development of the thalamocortical tract in *Pax6*$^{Sey/Sey}$↔*Pax6*$^{+/+}$ chimeric embryos

5.1 Introduction

In the previous two chapters the *Pax6*$^{Sey/Sey}$ and *Pax6*^{cKO} mouse models were used to examine the role that *Pax6* plays in the development of the thalamocortical tract. As has been discussed previously the *Pax6*$^{Sey/Sey}$ mouse has proved a useful tool for understanding the function of *Pax6*. But the wide ranging developmental defects seen in the *Pax6*$^{Sey/Sey}$ mouse can make interpretation of the phenotype difficult. Several conditional *Pax6* mutant mice have also been created using *Cre-lox* technology to knockout *Pax6* expression in specific regions of the developing brain. This approach has the advantage of knocking out *Pax6* in a targeted region while maintaining normal *Pax6* expression throughout the rest of the animal. There are, however, disadvantages associated with the use of *Cre-lox* technology. The deletion of the target gene will only occur after the activation of the Cre, which may be at a later developmental stage than when target gene expression begins. This would mean that there would be a delay before *Cre* mediated deletion will knockout the gene. This is of course the case for the *Pax6*^{cKO} described in chapter three, as *Pax6* expression begins in the embryo at E8.5 while *Gsh2* expression does not begin until a day later at E9.5 (Corbin et al., 2000; Walther and Gruss, 1991).

Another model system previously used to examine the function of *Pax6* is chimeric embryos which contain a mixture of *Pax6*$^{Sey/Sey}$ and WT cells. These embryos are created by injecting embryonic stem cells derived from *Pax6*$^{Sey/Sey}$ mice into WT blastocysts. When left develop these blastocysts form embryos that are composed of a mixture of WT and *Pax6*$^{Sey/Sey}$ cells. This system allows us to examine the behaviour of cells which have never expressed *Pax6* in a WT environment, or indeed WT cells in a *Pax6*$^{Sey/Sey}$ environment. A genetic marker such as the β-globin or Tau GFP transgene (Tg) is used to identify the mutant cells present in the chimera. Studies conducted using *Pax6*$^{Sey/Sey}$ *Tg*+↔*Pax6*$^{+/+}$ chimeras were initially important in elucidating which tissues require *Pax6* expression for their development. *Pax6*$^{Sey/Sey}$ cells are excluded from the retinal pigmented epithelium in the eye of *Pax6*$^{Sey/Sey}$↔*Pax6*$^{+/+}$ chimeras, and there are defects in the development of the lens

and optic cup. This demonstrates the vital role that *Pax6* plays in the development of the eye (Quinn et al., 1996).

In the forebrain of Pax6$^{Sey/Sey}$↔*Pax6*$^{+/+}$ chimeras, mutant cells are distributed normally throughout the MGE within the ventral telencephalon. In the thalamus however *Pax6*$^{Sey/Sey}$ and WT cells segregate into radial stripes of cells composed almost entirely of cells of one or the other genotype. This is in contrast to control *Pax6*$^{+/+}$*Tg*+↔*Pax6*$^{+/+}$, chimeras where cells both with the transgene and without are evenly distributed throughout the thalamus. This shows that *Pax6* is important for the development of the thalamus but is not required for the development of the MGE (Pratt et al., 2002). Observations have shown that the developing cortical plate of the *Pax6*$^{Sey/Sey}$ mouse is thinner than in the WT while the ventricular zone (VZ) and subventricular zone (SVZ) are much thicker (Caric et al., 1997). During normal cortical development neurons and neuronal precursors migrate radially from the VZ and SVZ along radial glial fibres to occupy positions in the cortical plate. In Pax6$^{Sey/Sey}$↔*Pax6*$^{+/+}$ chimeras, *Pax6*$^{Sey/Sey}$ cells are restricted to the ventricular zone and subventricular zone of the cortex and do not migrate radially into the cortical plate as WT cells do, while in control chimeras, cells carrying the transgene are distributed throughout the depth of the neocortex. This demonstrates that *Pax6* is required for the migration of neuronal precursors from the ventricular zone to the deeper layers of the developing cortex. The inability of progenitor cells lacking *Pax6* to migrate partly explains why the ventricular and subventricular zones are expanded in the *Pax6*$^{Sey/Sey}$ mouse (Talamillo et al., 2003).

The cortex of the *Pax6*$^{Sey/Sey}$ embryos becomes progressively ventralised during development, with the expression domain of ventral marker genes such as *Mash1* and *Dlx2* extending much further dorsally than in WT embryos (Kroll and O'Leary, 2005; Stoykova et al., 2000). From this observation alone, however, it is unclear whether *Pax6* is required in a cell autonomous fashion for the repression of ventral telencephalic cell fates and the adoption of dorsal telencephalic fates. In the cortex of Pax6$^{Sey/Sey}$↔*Pax6*$^{+/+}$ chimeras, *Pax6*$^{Sey/Sey}$ cells form clusters in a similar fashion to the stripes seen in the thalamus. These clusters of *Pax6*$^{Sey/Sey}$ cells do not express the transcription factor *Tbr2*, a marker for basal progenitor cells of the subventricular zone, while the WT cells surrounding the cluster express *Tbr2*

normally. In addition, *Pax6*$^{Sey/Sey}$ cells within the cortex express markers of ventral telencephalic cell fates *Mash1, Gsh2* and *Dlx2* in the absence of *Pax6*. This indicates a cell autonomous role for *Pax6* in repressing of ventral telencephalic cell fates and activating the expression of *Tbr2* (Quinn et al., 2007).

It is currently unclear how *Pax6* regulates axon guidance and what role it plays in the development of the thalamocortical tract. Two possible modes of action are: (*i*) *Pax6* is required in a cell autonomous manner by cells that project TCAs and controls the expression of guidance receptors and other genes required by the growth cone for guidance of the axon. (ii) *Pax6* expression influences cells along the route of the thalamocortical tract, regulating the expression of guidance cues and promoting pioneer axon growth in order to guide TCAs to the cortex. The thalamocortical tract of the *Pax6*$^{Sey/Sey}$ mouse does not form correctly; TCAs do not reach the telencephalon and a small number are misrouted to the hypothalamus (Pratt et al., 2002). Co-culture experiments where *Pax6*$^{Sey/Sey}$ or WT thalamus was cultured adjacent to WT ventral telencephalon showed that *Pax6*$^{Sey/Sey}$ TCAs were not guided through the ventral telencephalon normally as was the case for WT TCAs. This suggests that *Pax6* expression is required in the thalamus itself for correct TCA guidance (Pratt et al., 2000b).

Previous work has shown that *Slit/Robo* signalling is particularly important for the repulsion of TCAs from the hypothalamus (Bagri et al., 2002; Lopez-Bendito et al., 2007). In chapter three we saw that expression of guidance receptor *Robo2* is down regulated in the thalamus of *Pax6*$^{Sey/Sey}$ embryos while *Slit* expression is maintained at the hypothalamus. This suggests that *Pax6* is controlling TCA guidance in a cell autonomous fashion by promoting the expression of guidance receptor *Robo2* in cells that project TCAs. Another study has shown, however, that expression of attractive guidance cues in the cortex is altered in *Pax6*^{LacZKO} embryos (Jones et al., 2002). This suggests that *Pax6* is acting to regulate guidance cue expression in cells outside the thalamus to guide TCA growth cones to the cortex.

In this chapter the thalamocortical tract of *Pax6*$^{Sey/Sey}$↔*Pax6*$^{+/+}$ chimeras was examined in order to determine if *Pax6* is required cell autonomously for TCAs to reach the telencephalon. The hypothesis being that if *Pax6* expression in thalamic cells is an absolute requirement for TCAs to reach the telencephalon then *Pax6*$^{Sey/Sey}$

TCAs would not cross the DTB and would instead invade the hypothalamus as is the case in *Pax6*$^{Sey/Sey}$ embryos. Conversely if *Pax6* expression is not required cell autonomously then *Pax6*$^{Sey/Sey}$ TCAs should be visible within the telencephalon.

5.2 Results

5.2.1 *Pax6*$^{Sey/Sey}$ thalamocortical axons are able to contribute to the thalamocortical tract normally in *Pax6*$^{Sey/Sey}$↔*Pax6*$^{+/+}$ chimeras.

To investigate whether *Pax6* expression is required cell autonomously for TCAs to reach the telencephalon, *Pax6*$^{Sey/Sey}$↔*Pax6*$^{+/+}$ chimeras were created. These chimeras contain a mixture of WT and *Pax6*$^{Sey/Sey}$ cells. The chimeras were generated using an embryonic stem cell line derived from *Pax6*$^{Sey/Sey}$ mice. These stem cells ubiquitously express a Tau-GFP reporter (Pratt et al., 2000a); this means that *Pax6*$^{Sey/Sey}$ cells found in chimeras can be identified by their expression of GFP. The GFP has a cytoplasmic cellular localisation which is advantageous as it allows axons to be labelled in addition to cell bodies. This is in contrast to the previously used β-globin transgene reporter which was only present at the nucleus (Quinn et al., 2007).

To examine the behaviour of *Pax6*$^{Sey/Sey}$ TCAs in the chimeras immunohistochemistry was performed at E13.5 for L1 and GFP. E13.5 is shortly after TCAs have turned laterally and crossed the DTB to reach the MGE within the ventral telencephalon (Auladell et al., 2000). This stage was selected as it allows us to establish if *Pax6*$^{Sey/Sey}$ TCAs make their lateral turn and reach the ventral telencephalon. L1 is an axonal marker, used to label all TCAs be they *Pax6*$^{+/+}$ or *Pax6*$^{Sey/Sey}$; this will indicate the position of the thalamocortical tract in both chimeric and WT embryos. GFP will label all *Pax6*$^{Sey/Sey}$ cells and axons in the chimeras. In the WT control L1 can be seen labelling a thick bundle of TCAs leaving the thalamus, heading through the prethalamus and turning laterally towards the telencephalon (Fig. 1A, C, F). In the chimera stripes of GFP expressing cells can be seen in the thalamus (arrowheads, Fig. 1B), as has been previously described (Pratt et al., 2002). Despite this the forebrain of the chimera appears morphologically normal. An L1 labelled bundle of TCAs can also be seen extending from the thalamus and turning laterally towards the telencephalon as in the WT (Fig. 1B, D). A subset of these TCAs is also labelled with GFP (arrows, Fig 1B, E, G) indicating that these axons originate from *Pax6*$^{Sey/Sey}$ cells, and that the thalamocortical tract of the chimeras is composed of a mixture of *Pax6*$^{Sey/Sey}$ and *Pax6*$^{+/+}$ axons. This shows that

Pax6$^{Sey/Sey}$ axons are able to contribute to the thalamocortical tract normally and that *Pax6* expression is not required cell autonomously for TCAs to turn laterally towards the telencephalon.

5.2.2 *Pax6*$^{Sey/Sey}$ thalamocortical axons are able to reach the telencephalon in *Pax6*$^{Sey/Sey}$↔*Pax6*$^{+/+}$ chimeras.

At a further rostral level than that discussed in 5.2.1 L1 immunohistochemistry reveals a patch of TCAs within the ventral telencephalon of the WT (Fig 2A, C, F). At this developmental stage this patch of TCAs is at the leading edge of the thalamocortical tract as it extends towards the cortex. In the chimera this patch of TCAs can also be seen in a comparable position to that observed in the WT (Fig 2B, D). GFP immunohistochemistry shows that a subset of these TCAs along the medial edge of the patch expresses GFP and therefore originates from *Pax6*$^{Sey/Sey}$ cells (arrows, Fig. 2B, E, G). This demonstrates that TCAs from *Pax6*$^{Sey/Sey}$ cells are able to cross the DTB and reach the telencephalon; this is in contrast to the situation in *Pax6*$^{Sey/Sey}$ embryos where TCAs cannot cross the DTB or reach the telencephalon. This indicates that *Pax6* expression is not absolutely required in a cell autonomous fashion for the correct guidance of TCAs and that the *Pax6*$^{+/+}$ tissue through which the *Pax6*$^{Sey/Sey}$ TCAs grow is able to rescue the thalamocortical phenotype seen in the *Pax6*$^{Sey/Sey}$ mouse.

Table 1. A breakdown of the number of animals used in each of the experiments conducted as part of this chapter.

			n number	
Figure	Experiment	Age	WT	Chimera
1	*L1*/GFP immunohistochemistry	E13.5	2	3
2	*L1*/GFP immunohistochemistry	E13.5	2	3

***Figure 1. Pax6$^{Sey/Sey}$* TCAs can contribute to the thalamocortical tract in *Pax6$^{Sey/Sey}$*↔Pax6+/+ chimeras. (A,C,F)** L1 immunohistochemistry in WT brains at E13.5 reveals TCAs within the diencephalon. **(B,D,E,G)** Immunohistochemistry in *Pax6$^{Sey/Sey}$* ↔*Pax6*+/+ brains for GFP shows stripes of *Pax6$^{Sey/Sey}$* cells within the thalamus (arrowheads, B) and axons (B,E,G). L1 staining labels TCAs in the diencephalon turning towards the telencephalon (B,D,G). A subset of TCAs are positive for GFP (arrows E,G) indicating that *Pax6$^{Sey/Sey}$* TCAs can contribute to the thalamocortical tract and turn towards the telencephalon. Scale bars: A and B 500μm C-G 100μm.

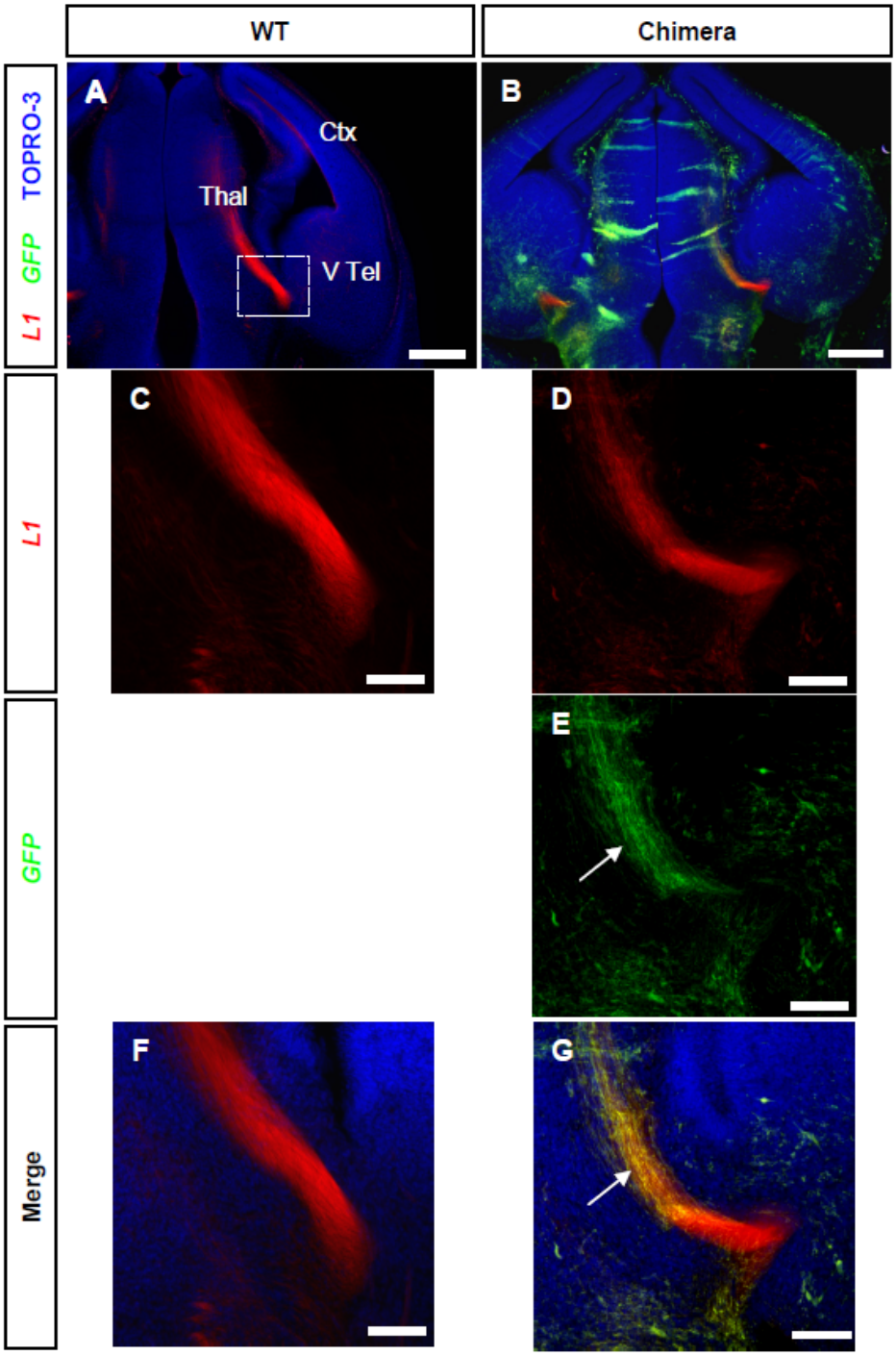
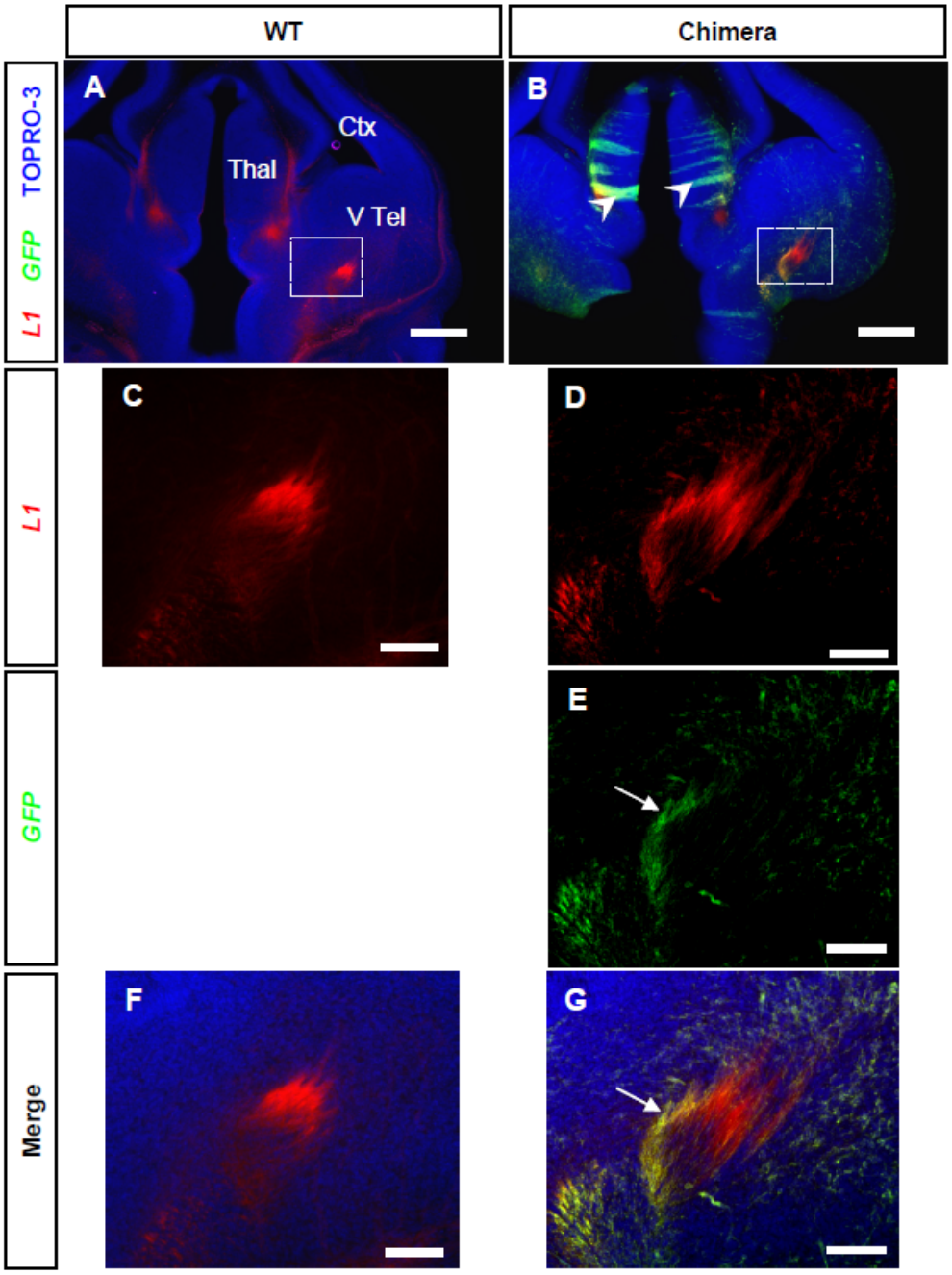

WT
Chimera
L1 GFP TOPRO-3
A
Ctx
Thal
V Tel
B
L1
C
D
GFP
E
Merge
F
G

***Fig 2. Pax6$^{Sey/Sey}$* TCAs can reach the ventral telencephalon normally in *Pax6$^{Sey/Sey}$*↔*Pax6$^{+/+}$* chimeras. (A,C,F)** L1 immunohistochemistry in WT brains at E13.5 reveals TCAs within the ventral telencephalon. **(B,D,E,G)** Immunohistochemistry in *Pax6$^{Sey/Sey}$*↔*Pax6$^{+/+}$* brains for GFP shows *Pax6$^{Sey/Sey}$* cells (arrowheads B) and axons (B,E,G). L1 staining labels a patch of TCAs in the ventral telencephalon (B,D,G). A subset of these TCAs are positive for GFP (arrows E,G) indicating that *Pax6$^{Sey/Sey}$* TCAs can reach the ventral telencephalon normally. Scale bars: A and B 500μm C-G 100μm.


WT
Chimera
A
B
Ctx
Thal
V Tel
TOPRO-3
GFP
L1
C
D
E
F
G
L1
GFP
Merge

5.3 Discussion

5.3.1 Summary

In this chapter it has been observed that in *Pax6*$^{Sey/Sey}$↔*Pax6*$^{+/+}$ chimeric embryos TCAs originating from *Pax6*$^{Sey/Sey}$ cells are able to contribute to the thalamocortical tract and reach the ventral telencephalon normally. This demonstrates that *Pax6* expression is not absolutely required cell-autonomously for TCAs to reach the telencephalon and suggests that *Pax6* acts in a non autonomous manner to set up axon guidance cues within the environment through which TCAs extend.

5.3.2 The cell-autonomous and non-autonomous role of *Pax6* in the guidance of thalamocortical axons

In this chapter the behaviour of *Pax6*$^{Sey/Sey}$ TCAs in a WT environment has been examined by using *Pax6*$^{Sey/Sey}$↔*Pax6*$^{+/+}$ chimeras which contain a mixture of *Pax6*$^{Sey/Sey}$ and *Pax6*$^{+/+}$ cells. Immunohistochemistry experiments have shown that *Pax6*$^{Sey/Sey}$ cells within the thalamus can extend TCAs. These TCAs contribute normally to the thalamocortical tract growing ventrally through the diencephalon and turning laterally to reach the ventral telencephalon. It cannot be completely ruled out that a small number of *Pax6*$^{Sey/Sey}$ TCAs invade the hypothalamus, as is the case in the *Pax6*$^{Sey/Sey}$ mouse, but it is clear that a significant number are guided normally. This finding indicates that *Pax6* expression is not absolutely required cell autonomously for TCAs to reach the telencephalon. Despite the fact that these cells have never expressed *Pax6* their axons are still able to be guided normally, at least as far as the ventral telencephalon. This is in stark contrast to the *Pax6*$^{Sey/Sey}$ mouse where TCAs do not reach the telencephalon at all (Pratt et al., 2002).

It appears that the *Pax6*$^{+/+}$ cells through which the *Pax6*$^{Sey/Sey}$ TCAs grow are able to rescue the phenotype seen in the *Pax6*$^{Sey/Sey}$ mouse. These *Pax6*$^{+/+}$ cells presumably provide guidance information to the growth cones of *Pax6*$^{Sey/Sey}$ TCAs as they grow through the diencephalon and the telencephalon, and these growth cones must have the ability to respond to this guidance. It is likely that the pioneer axon tracts that extend from the prethalamus and ventral telencephalon are intact in the

chimeras. These axons are important for the guidance of TCAs as far as the internal capsule (Molnar and Cordery, 1999). The action of these axons may in part explain why *Pax6*$^{Sey/Sey}$ TCAs are able to reach the telencephalon but their presence in the chimeras would need to be confirmed. *Pax6*$^{+/+}$ cells would also express axon guidance molecules important for TCA guidance, but it is unclear whether the *Pax6*$^{Sey/Sey}$ TCAs express guidance receptors at the growth cone. In chapter three we saw that expression of guidance receptor *Robo2* is markedly reduced in the thalamus of *Pax6*$^{Sey/Sey}$ embryos but it is unclear if there is a similar effect on *Pax6*$^{Sey/Sey}$ cells within the thalamus of the chimera. If the expression of guidance receptors, such as *Robo2,* is altered in the thalamic *Pax6*$^{Sey/Sey}$ cells then it would show that other guidance mechanisms such as those provided by pioneer axons are sufficient for TCAs to reach the telencephalon.

Although it has not been shown that pioneer axons from the prethalamus and ventral telencephalon are definitely present in the chimeras, we do know that *Pax6*$^{+/+}$ TCAs are present alongside *Pax6*$^{Sey/Sey}$ TCAs within the thalamocortical tract. Some of the earliest research into the mechanisms of axon guidance in grasshoppers showed how the first axons which extend from neurons within the limb bud are able to 'pioneer' the axon tract and guide later forming axons (Bate, 1976). There is evidence that descending cortical axons may be important for the guidance of TCAs from the internal capsule zone to the cortex (Metin and Godement, 1996; Molnar et al., 1998a). In light of this it would be reasonable to assume that TCAs within the thalamocortical tract can provide guidance for each other. The presence of *Pax6*$^{+/+}$ TCAs within the thalamocortical tract may be providing this guidance by acting as a scaffold on which the *Pax6*$^{Sey/Sey}$ TCAs are able to grow. It could be that the presence of these TCAs is the primary cause of the rescue of the *Pax6*$^{Sey/Sey}$ phenotype rather than the *Pax6*$^{+/+}$ environment through which the TCAs grow.

The co-culture experiments by Pratt et al (2000) showed that *Pax6*$^{Sey/Sey}$ TCAs were unable to grow into explants of WT ventral telencephalon or cortex in a manner that mimicked the thalamocortical tract, while *Pax6*$^{+/+}$ TCAs were able to do this. This demonstrated that *Pax6*$^{Sey/Sey}$ TCAs were unable to respond to the axon guidance cues present in the WT ventral telencephalon or cortex. This implies that *Pax6* expression is required during the development of the thalamus to confer on

TCAs the ability to respond to these guidance cues. The data in this chapter challenges the idea that *Pax6* expression is absolutely required in the thalamus for correct TCA guidance but it does not rule out a cell autonomous role for *Pax6*. As has been discussed above the $Pax6^{+/+}$ TCAs present in the chimeras may provide guidance to $Pax6^{Sey/Sey}$ TCAs. *Pax6* expression may be required for the first $Pax6^{+/+}$ TCAs to reach the telencephalon by influencing the expression of guidance receptors at the growth cone. The $Pax6^{+/+}$ TCAs were not present in the co-culture experiments and this may be one reason why we see different results from the two sets of experiments. We know that the prethalamus can play an important role in axon guidance (Andrews and Mastick, 2003; Mitrofanis and Baker, 1993), this structure was absent from the co-culture experiments but was present in the chimeras. The fact that guidance of $Pax6^{Sey/Sey}$ TCAs was more correct in the presence of the prethalamus provides further evidence that this region is important for the guidance of TCAs.

While it is clear that *Pax6* is heavily involved in axon guidance of the thalamocortical tract, it is still unclear what its exact mode of action is. This chapter provides evidence that *Pax6* expression influences the tissues surrounding the thalamocortical tract in order to guide TCAs. This does not rule out a cell autonomous role for *Pax6* in the thalamus itself as suggested by previous studies and data from chapter three. Therefore it appears likely that *Pax6* regulates thalamocortical development by acting both on thalamic cells that extend TCAs and on other cells along the route of the tract such as those in the prethalamus and ventral telencephalon.

Chapter 6: Discussion

6.1 Summary

The aim of this thesis was to examine the role that transcription factor *Pax6* plays in the development of the thalamocortical tract. To do this three different mouse models have been used to determine in what ways *Pax6* influences the guidance of thalamocortical axons (TCAs) and in which cell populations *Pax6* exerts this influence.

In chapter three the Small eye ($Pax6^{Sey/Sey}$) mouse was used to examine TCA guidance in embryos which completely lack *Pax6*. Tract tracing experiments showed conclusively that TCAs cannot reach the telencephalon in these embryos and that a small number of TCAs are misrouted to the hypothalamus, a region normally repulsive to thalamocortical axons. The large axon tract which was observed in the ventral telencephalon of $Pax6^{Sey/Sey}$ embryos by this and other studies (Hevner et al., 2002; Jones et al., 2002; Kawano et al., 1999) was shown to be composed of axons which originate within the ventral telencephalon itself rather than TCAs originating from the thalamus. Analysis of the expression patterns of *Slit* and *Robo* guidance cues, which are primarily responsible for the repulsive nature of the hypothalamus (Bagri et al., 2002; Braisted et al., 2009), revealed that expression of receptor *Robo2* was reduced in the thalamus of $Pax6^{Sey/Sey}$ embryos. This may explain why TCAs invade the hypothalamus in these embryos and provides further evidence that *Pax6* acts to regulate the expression of guidance cues required for thalamocortical development.

In chapter four transgenic embryos were created in which *Pax6* expression was specifically reduced in the prethalamus and ventral telencephalon. This allowed me to determine if *Pax6* expression was required within these cells for the guidance of TCAs, and if so by what mechanism *Pax6* influenced this guidance. Examination of thalamocortical development in these embryos revealed that although a large number of TCAs were able to reach the cortex normally, some TCAs became disorganised or abnormally fasciculated within the thalamus and prethalamus. This demonstrates that *Pax6* expression is required at the prethalamus for the guidance of TCAs through the diencephalon. Analysis of the outgrowth of prethalamic pioneer

axons, which are thought to guide TCAs from the thalamus into the prethalamus, showed that fewer axons were present in the mutant embryos compared to control. This loss of pioneer axons is consistent with the disruption of TCA guidance in the thalamus and suggests that *Pax6* expression is required for the development of pioneer axon tracts.

In chapter five chimeric embryos which were composed of a mixture of $Pax6^{Sey/Sey}$ and wild type cells were analysed. Examination of the thalamocortical tract in these embryos demonstrated that TCAs from cells lacking *Pax6* are able to reach the ventral telencephalon normally. This shows that *Pax6* expression is not absolutely required for TCAs to reach the ventral telencephalon.

In this chapter I will discuss how the results in this thesis add to current understanding of the role that *Pax6* plays in the development of the thalamocortical tract, and the prospects for further research in this area.

6.2 Discussion

6.2.1 The regulation of axon guidance cues by *Pax6*

Analysis of *Pax6* loss of function mutant embryos has provided evidence that *Pax6* may regulate the expression of axon guidance cues important for the development of the thalamocortical tract. The study by Jones et al, (2002) showed that the expression of attractive *Semaphorin* guidance cues, *Sema3C* and *Sema5A*, are reduced in the cortex of $Pax6^{LacZKO}$ embryos. This suggests that *Pax6* may promote the expression of these guidance cues. Tract tracing experiments from chapter three of this thesis show that TCAs do not reach the telencephalon in $Pax6^{Sey/Sey}$ embryos, so it is unlikely that this change in *Semaphorin* expression is a cause of the TCA phenotype seen in these embryos. There is also evidence that the expression of *Netrin-1* is up-regulated in the thalamus of $Pax6^{Sey/Sey}$ embryos (Tsuchiya et al., 2009) which again implies that *Pax6* may regulate guidance cue expression in order to control axon guidance.

In chapter three it was shown that the guidance cue receptor *Robo2* is down-regulated in the thalamus of $Pax6^{Sey/Sey}$ embryos. *Robo2* is a receptor for the repulsive guidance cues *Slit1* and *Slit2*. *Slit/Robo* signalling is responsible for the repulsion of TCAs from the hypothalamus and is important for the turning of TCAs towards the telencephalon (Bagri et al., 2002; Braisted et al., 2009; Lopez-Bendito et al., 2007). The loss of *Robo2* in thalamic cells may be a reason for some TCAs invading the hypothalamus in these embryos. This suggests that *Pax6* may promote the expression of *Robo2* in thalamic cells. *Robo2* expression also appears reduced in the cortex of $Pax6^{Sey/Sey}$ embryos (where *Pax6* is normally expressed) but expression appears roughly normal at the ventral telencephalon (where *Pax6* is not expressed) which is further suggestive that *Robo2* is *Pax6* regulated. A recent study has shown that the LIM-homeodomain transcription factor *Lhx2* controls TCA guidance by regulation of both *Robo1* and *Robo2*. In particular they show that *Lhx2* represses *Robo1* and *Robo2* expression in thalamic neurons, and that when *Lhx2* is overexpressed in the thalamus TCAs are promoted to enter the hypothalamus rather than turn towards the telencephalon. *Lhx2* is able to bind to regions within the *Robo1* and *Robo2* regulatory

sequences which demonstrates that *Lhx2* acts as a direct transcriptional regulator of the *Robo* genes (Marcos-Mondejar et al., 2012). From the results in this thesis it is unclear whether *Pax6* directly regulates *Robo2* expression in the same manner as *Lhx2*. To test this, a bioinformatic approach could be used to search for putative *Pax6* binding sequences within the regulatory elements of the *Robo2* gene, while a chromatin immunoprecipitation (ChIP) assay could be used to determine if *Pax6* is able to bind to these putative binding sites.

6.2.2 The importance of *Pax6* expression within the prethalamus for thalamocortical tract development

As TCAs extend towards the cortex, the first structure they encounter upon leaving the thalamus is the prethalamus. Several previous studies have provided evidence that the prethalamus is an important intermediate target for TCAs. This is due to the pioneer axons which grow from the prethalamus and are proposed to guide TCAs from the thalamus across the ZLI into the prethalamus (Braisted et al., 1999; Mitrofanis and Baker, 1993; Molnar and Cordery, 1999).

Pax6 is strongly expressed within the embryonic prethalamus in both postmitotic neurons and neuronal progenitors. To determine if *Pax6* expression is required at this position for TCA guidance, conditional *Pax6* knockout ($Pax6^{cKO}$) embryos were generated in which *Pax6* expression was specifically reduced in the prethalamus. In these embryos some TCAs become disorganised, with axons forming bundles which project aberrantly within the thalamus and prethalamus. This shows that *Pax6* expression is required at the prethalamus for the normal thalamocortical development. It is interesting that the TCA guidance within the thalamus is affected as *Pax6* expression is normal within this region. An explanation for this comes from the fact that the number of prethalamic pioneer axons reaching the thalamus is reduced in these embryos, which may cause TCA guidance errors. This suggests that *Pax6* expression is required for the development of this pioneer axon tract. The disruption of TCA guidance within the thalamus itself is seen in $Pax6^{Sey/Sey}$ embryos and in embryos deficient for transcription factor *Mash1,* with TCAs forming dense bundles in the thalamus of both mutants (Chapter Three)(Tuttle et al., 1999). In *Mash1* mutant embryos the prethalamic pioneer axon are also disrupted as in the

Pax6^{cKO}. The association between pioneer axon disruption and TCA guidance errors seen in *Pax6*^{cKO} embryos provides some of the best evidence yet that these pioneer axons are essential for the TCA guidance within the diencephalon.

It appears that *Pax6* is required for the normal development of these pioneer axons but the molecular mechanism by which *Pax6* controls this process remains unclear. Examination of *Pax6*^{cKO} embryos in chapter four has given little insight into the molecular cause of the disrupted pioneer axon growth seen in these embryos. The molecular patterning of the diencephalon in *Pax6*^{cKO} embryos seems to be largely unchanged which means it is unlikely that the prethalamic cells that extend pioneer axons are respecified. There is also no increase in the rate of cell death within the prethalamus which discounts the possibility that these cells have simply died, therefore reducing pioneer axon number. In order to further understand how *Pax6* influences the growth of pioneer axons it would be important to work out which genes *Pax6* may regulate in the prethalamic cells which extend these axons. One candidate gene which may be of interest is *R-cadherin*. Previous studies have shown that *Pax6*-regulated cell adhesion molecule *R-cadherin* acts as a growth promoting cue for pioneer axon growth (Andrews and Mastick, 2003). *R-cadherin* expression appears unaltered in *Pax6*^{cKO} embryos however which makes it unlikely that a change in *R-cadherin* expression is responsible for reduced pioneer axon growth, in this population of pioneers at least. In addition to cell adhesion molecules other candidate genes include axon guidance cues, particularly those which may be expressed at the growth cone of pioneer axons such as *Robos, Semaphorins* and *Neuropillins.*

6.2.3 The importance of *Pax6* expression within the ventral telencephalon for thalamocortical tract development

Pax6 is highly expressed throughout the progenitor cells of the dorsal telencephalon, but is expressed at much lower levels within the ventral telencephalon in progenitor cells and a population of cells close to the amygdaloid region. In this study I have attempted to understand what role (if any) *Pax6* expression at this position plays in the guidance of TCAs. In chapter three it was observed that the axon permissive 'corridor' which guides TCAs through the ventral telencephalon

(Lopez-Bendito et al., 2006) develops highly abnormally in *Pax6*$^{Sey/Sey}$ embryos. The lack of an axon permissive corridor may contribute to the inability of TCAs to enter the ventral telencephalon in these animals. This is consistent with the previous finding that the migration of corridor cells from the LGE to the MGE is disrupted in embryos where *Pax6* expression has been depleted in cells close to the amygdaloid region, although this disruption is much less severe than that seen in the *Pax6*$^{Sey/Sey}$ embryo (Simpson et al., 2009).

In chapter four *Pax6*^{cKO} embryos were analysed in which *Pax6* expression was reduced at the ventricular zone of the LGE; it is from this population of cells that the corridor cells will migrate into the MGE. Analysis of the corridor in these embryos showed that the corridor forms normally and that TCAs navigate normally through the ventral telencephalon. This does not necessarily mean that *Pax6* expression is not required in these cells for their migration, but that the deletion of *Pax6* in these cells at the specific time point that the *Gsh2*^{Cre} is active is not sufficient to affect cell migration. To further investigate whether *Pax6* is required cell-autonomously for corridor cell migration a different *Cre* strain may be needed to drive *Pax6* deletion. One possibility is the *Mash1*^{Cre} which expresses *Cre* throughout the progenitor cells of the ventral telencephalon (Battiste et al., 2007). This may promote a more widespread deletion of *Pax6* in migrating corridor cells at an earlier stage; this would then allow us to determine if *Pax6* expression was necessary for these cells to migrate.

6.2.4 The cell-autonomous nature of *Pax6* in the guidance of thalamocortical axons

The study of several different *Pax6* mutant mice has demonstrated that *Pax6* expression is required for the development of the thalamocortical tract. The question of whether or not *Pax6* expression is required cell autonomously for normal TCA guidance remains unanswered definitively. The previous sections discuss the manner in which *Pax6* expression in cells outside the thalamus can influence TCA guidance but evidence suggests that *Pax6* expression within thalamic neurons themselves is also of great importance for thalamocortical development.

A likely way by which transcription factors like *Pax6* may control TCA guidance is to modulate the expression of guidance receptors in thalamic cells. The results from chapter three showed that *Pax6* may regulate the expression of *Robo2*. This suggests that *Pax6* can act in this cell autonomous manner to control the ability of the TCA growth cone to respond to different molecular cues found along their route. The co-culture experiments by Pratt et al (2000) showed that $Pax6^{Sey/Sey}$ TCAs were unable to respond normally when confronted with WT ventral telencephalon demonstrating that *Pax6* expression is required in thalamic cells for normal TCA guidance. This may be due to a change in the expression level of guidance receptors on the growth cone, including *Robo2*. Conversely, the results from chapter five showed that in chimeric embryos $Pax6^{Sey/Sey}$ TCAs are able to grow through a WT environment to reach the ventral telencephalon. This suggests that these axons are in fact able to respond to guidance cues normally (as far as the ventral telencephalon at least). This result though is complicated by the presence of WT TCAs; fasciculation with these WT TCAs may be sufficient to rescue the defective guidance of the $Pax6^{Sey/Sey}$ TCAs.

It appears highly likely that *Pax6* expression is required within the thalamus itself for the guidance of TCAs. To prove this conclusively, conditional mutagenesis could be used to delete *Pax6* expression specifically throughout the thalamus while maintaining *Pax6* expression elsewhere. The $Gbx2^{Cre}$ mouse could possibly be used to accomplish this (Chen et al., 2009). The ability or inability of the TCAs to reach the cortex normally in this hypothetical mutant embryo would demonstrate whether or not *Pax6* is required in the thalamus. Analysis of any molecular changes which may arise in the thalamus of these mutants would also give some insight in to the cell-autonomous mechanism by which *Pax6* may regulate axon guidance.

6.2.5 Summary

The findings of this thesis have expanded current knowledge of thalamocortical tract development and axon guidance in general in several ways. In particular we have seen further evidence of the role that transcription factors play in the control of axon guidance including regulation of guidance cue expression. We

have also seen the importance of fasciculation during thalamocortical tract development.

Some of the earliest studies of axon guidance which examined the navigation of axons within the grasshopper limb bud identified that axon-axon interaction was a powerful method by which axons are guided during development (Bentley and Keshishian, 1982). Fasciculation is also an important axon guidance mechanism within the central and peripheral nervous system of vertebrate organisms. It has been proposed that the fasciculation of TCAs with pioneer axons and descending cortical axons is required for TCAs to reach the cortex (Molnar et al., 1998a). In chapter 5 we have seen that when the development of these pioneers is disturbed, so too is the guidance of TCAs, suggesting that fasciculation of TCAs with these pioneers is required for normal TCA guidance. This is consistent with a growing body of evidence which shows that axon fasciculation is of vital importance for reciprocal connectivity between the thalamus and cortex. Recent studies have shown that the presence of descending cortical axons is necessary for TCAs to reach the cortex (Chen et al., 2012) and likewise the presence of TCAs is also necessary for the guidance of corticothalamic axons (Deck et al., 2013).

From the experiments in this thesis the molecular basis of TCA/pioneer interaction remains unclear, a likely scenario is that ligands expressed by one set of axons bind to receptors expressed by the other. These ligand/receptor pairs could be axon guidance molecules such as *Semaphorins* and *Neuropilins* alternatively they may be cell adhesion molecules such as *Cadherins*. Research examining the guidance of cranial nerve axons as they leave the hindbrain has identified that the absence of *Sema3A* expression results in abnormal defasciculation of these axons (Taniguchi et al., 1997). Likewise the cell adhesion molecules *Cadherin6B* and *Cadherin7* control cranial nerve axon fasciculation, for example *Cadherin6B* gain of function causes axon hyper-fasciculation while loss of function causes defasciculation (Barnes et al., 2010). It is possible that the expression of combinations of *Cadherins* or other cell adhesion molecules is required for fasciculation of prethalamic pioneer axons with TCAs; an analysis of *Cadherin* expression on these axons may indicate which genes are particularly important for this process.

Research conducted over the last 15 years has shown that certain developmentally important transcription factors are required for the formation of major axon tracts within the brain including the thalamocortical tract. This includes transcription factors such as *Pax6, Emx2, Gbx2, Mash1* and *Gli3* (Hevner et al., 2002; Lopez-Bendito et al., 2002; Magnani et al., 2010; Tuttle et al., 1999). More recent work however has begun to show not only that these transcription factors are necessary for normal thalamocortical development but revealed the mechanism by which transcription factors influence TCA guidance. This includes the regulation of expression of axon guidance molecules. In this thesis there is evidence that *Pax6* regulates *Robo2* expression within the thalamus. Several recent studies have shown that precise regulation of *Robo* genes is of vital importance for correct TCA guidance and growth. LIM-homeodomain transcription factors *Lhx2* and *Lhx9* have been shown to directly regulate *Robo1* and *2*, while *Gbx2* in turn regulates *Lhx2* and *Lhx9* (Chatterjee et al., 2012; Marcos-Mondejar et al., 2012). There is also evidence that *Robo1* expression is transcriptionally regulated by spontaneous activity via Calcium sensitive transcription factor NF-κB (Mire et al., 2012). Whether *Pax6* regulates *Robo* expression indirectly via other transctiption factors such as the Lhx genes, or directly targets *Robo2* is unclear. The evidence from this thesis underlines the importance of the regulation of Robo gene expression by transcription factors for the guidance of thalamocortical axons.

6.2.6 Conclusion

The guidance of TCAs from the thalamus, through the ventral telencephalon to their target in the cortex is a complex process that involves the regulated expression of a host of different genes which may directly or indirectly affect axon growth. The control and co-ordination of this process is likely to be achieved by transcription factors which act to modulate the expression of other genes. *Pax6* is just one transcription factor which previous studies have shown to be involved in the formation of the thalamocortical tract.

Evidence from this and other studies allows for the construction of a hypothetical model by which *Pax6* may regulate thalamocortical development. Firstly *Pax6* expression within the thalamic neurons (or their progenitors) promotes the expression of guidance receptors, such as *Robo2*, at the growth cone of TCAs. This acts to 'program' TCA growth cones to respond to particular molecular cues found along the route of the thalamocortical tract. Secondly, *Pax6* expression within the prethalamus promotes the growth of pioneer axons from the prethalamus into the thalamus. These axons assist in the guidance of TCAs as they extend from the thalamus into the prethalamus crossing the ZLI. Thirdly *Pax6* expression within the ventral telencephalon regulates the migration of cells from the LGE to the MGE to form the axon permissive corridor. The formation of the corridor is required for TCAs to cross the DTB and proceed normally through the ventral telencephalon. Further research will be required to uncover the precise molecular mechanisms by which *Pax6* controls these cellular processes which influence TCA guidance.

Bibliography

Ahmed, G., Shinmyo, Y., Ohta, K., Islam, S. M., Hossain, M., Naser, I. B., Riyadh, M. A., Su, Y., Zhang, S., Tessier-Lavigne, M. et al. (2011). Draxin inhibits axonal outgrowth through the netrin receptor DCC. *J Neurosci* **31**, 14018-23.

Aletta, J. M. and Greene, L. A. (1988). Growth cone configuration and advance: a time-lapse study using video-enhanced differential interference contrast microscopy. *J Neurosci* **8**, 1425-35.

Andrews, G. L. and Mastick, G. S. (2003). R-cadherin is a Pax6-regulated, growth-promoting cue for pioneer axons. *J Neurosci* **23**, 9873-80.

Auladell, C., Perez-Sust, P., Super, H. and Soriano, E. (2000). The early development of thalamocortical and corticothalamic projections in the mouse. *Anat Embryol (Berl)* **201**, 169-79.

Bagnard, D., Chounlamountri, N., Puschel, A. W. and Bolz, J. (2001). Axonal surface molecules act in combination with semaphorin 3a during the establishment of corticothalamic projections. *Cereb Cortex* **11**, 278-85.

Bagri, A., Marin, O., Plump, A. S., Mak, J., Pleasure, S. J., Rubenstein, J. L. and Tessier-Lavigne, M. (2002). Slit proteins prevent midline crossing and determine the dorsoventral position of major axonal pathways in the mammalian forebrain. *Neuron* **33**, 233-48.

Barnes, S. H., Price, S. R., Wentzel, C. and Guthrie, S. C. (2010). Cadherin-7 and cadherin-6B differentially regulate the growth, branching and guidance of cranial motor axons. *Development* **137**, 805-14.

Bashaw, G. J., Kidd, T., Murray, D., Pawson, T. and Goodman, C. S. (2000). Repulsive axon guidance: Abelson and Enabled play opposing roles downstream of the roundabout receptor. *Cell* **101**, 703-15.

Bastiani, M. J., du Lac, S. and Goodman, C. S. (1986). Guidance of neuronal growth cones in the grasshopper embryo. I. Recognition of a specific axonal pathway by the pCC neuron. *J Neurosci* **6**, 3518-31.

Bate, C. M. (1976). Pioneer neurones in an insect embryo. *Nature* **260**, 54-6.

Battiste, J., Helms, A. W., Kim, E. J., Savage, T. K., Lagace, D. C., Mandyam, C. D., Eisch, A. J., Miyoshi, G. and Johnson, J. E. (2007). Ascl1 defines sequentially generated lineage-restricted neuronal and oligodendrocyte precursor cells in the spinal cord. *Development* **134**, 285-93.

Bentley, D. and Caudy, M. (1983). Pioneer axons lose directed growth after selective killing of guidepost cells. *Nature* **304**, 62-5.

Bentley, D. and Keshishian, H. (1982). Pathfinding by peripheral pioneer neurons in grasshoppers. *Science* **218**, 1082-8.

Bielle, F., Marcos-Mondejar, P., Keita, M., Mailhes, C., Verney, C., Nguyen Ba-Charvet, K., Tessier-Lavigne, M., Lopez-Bendito, G. and Garel, S. (2011a). Slit2 activity in the migration of guidepost neurons shapes thalamic projections during development and evolution. *Neuron* **69**, 1085-98.

Bielle, F., Marcos-Mondejar, P., Leyva-Diaz, E., Lokmane, L., Mire, E., Mailhes, C., Keita, M., Garcia, N., Tessier-Lavigne, M., Garel, S. et al. (2011b). Emergent growth cone responses to combinations of Slit1 and Netrin 1 in thalamocortical axon topography. *Curr Biol* **21**, 1748-55.

Bishop, K. M., Rubenstein, J. L. and O'Leary, D. D. (2002). Distinct actions of Emx1, Emx2, and Pax6 in regulating the specification of areas in the developing neocortex. *J Neurosci* **22**, 7627-38.

Blakemore, C. and Molnar, Z. (1990). Factors involved in the establishment of specific interconnections between thalamus and cerebral cortex. *Cold Spring Harb Symp Quant Biol* **55**, 491-504.

Bonnin, A., Torii, M., Wang, L., Rakic, P. and Levitt, P. (2007). Serotonin modulates the response of embryonic thalamocortical axons to netrin-1. *Nat Neurosci* **10**, 588-97.

Boretius, S., Michaelis, T., Tammer, R., Ashery-Padan, R., Frahm, J. and Stoykova, A. (2009). In vivo MRI of altered brain anatomy and fiber connectivity in adult pax6 deficient mice. *Cereb Cortex* **19**, 2838-47.

Braisted, J. E., Catalano, S. M., Stimac, R., Kennedy, T. E., Tessier-Lavigne, M., Shatz, C. J. and O'Leary, D. D. (2000). Netrin-1 promotes thalamic axon growth and is required for proper development of the thalamocortical projection. *J Neurosci* **20**, 5792-801.

Braisted, J. E., Ringstedt, T. and O'Leary, D. D. (2009). Slits are chemorepellents endogenous to hypothalamus and steer thalamocortical axons into ventral telencephalon. *Cereb Cortex* **19 Suppl 1**, i144-51.

Braisted, J. E., Tuttle, R. and O'Leary D, D. (1999). Thalamocortical axons are influenced by chemorepellent and chemoattractant activities localized to decision points along their path. *Dev Biol* **208**, 430-40.

Brose, K., Bland, K. S., Wang, K. H., Arnott, D., Henzel, W., Goodman, C. S., Tessier-Lavigne, M. and Kidd, T. (1999). Slit proteins bind Robo receptors and have an evolutionarily conserved role in repulsive axon guidance. *Cell* **96**, 795-806.

Cajal, S. R. Y. (1890). A quelle epoque apparaissent les expansions des cellule nerveuses de la moelle epinere du poulet. *Anatomomischner Anzeiger* **5**, 609-613.

Caric, D., Gooday, D., Hill, R. E., McConnell, S. K. and Price, D. J. (1997). Determination of the migratory capacity of embryonic cortical cells lacking the transcription factor Pax-6. *Development* **124**, 5087-96.

Chapouton, P., Gartner, A. and Gotz, M. (1999). The role of Pax6 in restricting cell migration between developing cortex and basal ganglia. *Development* **126**, 5569-79.

Chatterjee, M., Li, K., Chen, L., Maisano, X., Guo, Q., Gan, L. and Li, J. Y. (2012). Gbx2 regulates thalamocortical axon guidance by modifying the LIM and Robo codes. *Development* **139**, 4633-43.

Chen, H., Chedotal, A., He, Z., Goodman, C. S. and Tessier-Lavigne, M. (1997). Neuropilin-2, a novel member of the neuropilin family, is a high affinity receptor for the semaphorins Sema E and Sema IV but not Sema III. *Neuron* **19**, 547-59.

Chen, L., Guo, Q. and Li, J. Y. (2009). Transcription factor Gbx2 acts cell-nonautonomously to regulate the formation of lineage-restriction boundaries of the thalamus. *Development* **136**, 1317-26.

Chen, Y., Magnani, D., Theil, T., Pratt, T. and Price, D. J. (2012). Evidence that descending cortical axons are essential for thalamocortical axons to cross the pallial-subpallial boundary in the embryonic forebrain. *PLoS One* **7**, e33105.

Cheng, H. J., Nakamoto, M., Bergemann, A. D. and Flanagan, J. G. (1995). Complementary gradients in expression and binding of ELF-1 and Mek4 in development of the topographic retinotectal projection map. *Cell* **82**, 371-81.

Cocas, L. A., Georgala, P. A., Mangin, J. M., Clegg, J. M., Kessaris, N., Haydar, T. F., Gallo, V., Price, D. J. and Corbin, J. G. (2011). Pax6 is required at the telencephalic pallial-subpallial boundary for the generation of neuronal diversity in the postnatal limbic system. *J Neurosci* **31**, 5313-24.

Corbin, J. G., Gaiano, N., Machold, R. P., Langston, A. and Fishell, G. (2000). The Gsh2 homeodomain gene controls multiple aspects of telencephalic development. *Development* **127**, 5007-20.

Corbin, J. G., Rutlin, M., Gaiano, N. and Fishell, G. (2003). Combinatorial function of the homeodomain proteins Nkx2.1 and Gsh2 in ventral telencephalic patterning. *Development* **130**, 4895-906.

Cordery, P. and Molnar, Z. (1999). Embryonic development of connections in turtle pallium. *J Comp Neurol* **413**, 26-54.

De Carlos, J. A. and O'Leary, D. D. (1992). Growth and targeting of subplate axons and establishment of major cortical pathways. *J Neurosci* **12**, 1194-211.

Deck, M., Lokmane, L., Chauvet, S., Mailhes, C., Keita, M., Niquille, M., Yoshida, M., Yoshida, Y., Lebrand, C., Mann, F. et al. (2013). Pathfinding of

corticothalamic axons relies on a rendezvous with thalamic projections. *Neuron* **77**, 472-84.

Dent, E. W., Gupton, S. L. and Gertler, F. B. (2011). The growth cone cytoskeleton in axon outgrowth and guidance. *Cold Spring Harb Perspect Biol* **3**.

Drescher, U., Kremoser, C., Handwerker, C., Loschinger, J., Noda, M. and Bonhoeffer, F. (1995). In vitro guidance of retinal ganglion cell axons by RAGS, a 25 kDa tectal protein related to ligands for Eph receptor tyrosine kinases. *Cell* **82**, 359-70.

Dufour, A., Egea, J., Kullander, K., Klein, R. and Vanderhaeghen, P. (2006). Genetic analysis of EphA-dependent signaling mechanisms controlling topographic mapping in vivo. *Development* **133**, 4415-20.

Dufour, A., Seibt, J., Passante, L., Depaepe, V., Ciossek, T., Frisen, J., Kullander, K., Flanagan, J. G., Polleux, F. and Vanderhaeghen, P. (2003). Area specificity and topography of thalamocortical projections are controlled by ephrin/Eph genes. *Neuron* **39**, 453-65.

Elkins, T., Zinn, K., McAllister, L., Hoffmann, F. M. and Goodman, C. S. (1990). Genetic analysis of a Drosophila neural cell adhesion molecule: interaction of fasciclin I and Abelson tyrosine kinase mutations. *Cell* **60**, 565-75.

Englund, C., Fink, A., Lau, C., Pham, D., Daza, R. A., Bulfone, A., Kowalczyk, T. and Hevner, R. F. (2005). Pax6, Tbr2, and Tbr1 are expressed sequentially by radial glia, intermediate progenitor cells, and postmitotic neurons in developing neocortex. *J Neurosci* **25**, 247-51.

Erskine, L., Williams, S. E., Brose, K., Kidd, T., Rachel, R. A., Goodman, C. S., Tessier-Lavigne, M. and Mason, C. A. (2000). Retinal ganglion cell axon guidance in the mouse optic chiasm: expression and function of robos and slits. *J Neurosci* **20**, 4975-82.

Figdor, M. C. and Stern, C. D. (1993). Segmental organization of embryonic diencephalon. *Nature* **363**, 630-4.

Flames, N., Long, J. E., Garratt, A. N., Fischer, T. M., Gassmann, M., Birchmeier, C., Lai, C., Rubenstein, J. L. and Marin, O. (2004). Short- and long-range attraction of cortical GABAergic interneurons by neuregulin-1. *Neuron* **44**, 251-61.

Fode, C., Ma, Q., Casarosa, S., Ang, S. L., Anderson, D. J. and Guillemot, F. (2000). A role for neural determination genes in specifying the dorsoventral identity of telencephalic neurons. *Genes Dev* **14**, 67-80.

Gallo, G. and Letourneau, P. C. (2004). Regulation of growth cone actin filaments by guidance cues. *J Neurobiol* **58**, 92-102.

Gao, P. P., Zhang, J. H., Yokoyama, M., Racey, B., Dreyfus, C. F., Black, I. B. and Zhou, R. (1996). Regulation of topographic projection in the brain: Elf-1 in the hippocamposeptal system. *Proc Natl Acad Sci U S A* **93**, 11161-6.

Garel, S., Yun, K., Grosschedl, R. and Rubenstein, J. L. (2002). The early topography of thalamocortical projections is shifted in Ebf1 and Dlx1/2 mutant mice. *Development* **129**, 5621-34.

Georgala, P. A., Manuel, M. and Price, D. J. (2011). The generation of superficial cortical layers is regulated by levels of the transcription factor Pax6. *Cereb Cortex* **21**, 81-94.

Glaser, T., Jepeal, L., Edwards, J. G., Young, S. R., Favor, J. and Maas, R. L. (1994). PAX6 gene dosage effect in a family with congenital cataracts, aniridia, anophthalmia and central nervous system defects. *Nat Genet* **7**, 463-71.

Grindley, J. C., Hargett, L. K., Hill, R. E., Ross, A. and Hogan, B. L. (1997). Disruption of PAX6 function in mice homozygous for the Pax6Sey-1Neu mutation produces abnormalities in the early development and regionalization of the diencephalon. *Mech Dev* **64**, 111-26.

Guillemot, F. and Joyner, A. L. (1993). Dynamic expression of the murine Achaete-Scute homologue Mash-1 in the developing nervous system. *Mech Dev* **42**, 171-85.

Heins, N., Malatesta, P., Cecconi, F., Nakafuku, M., Tucker, K. L., Hack, M. A., Chapouton, P., Barde, Y. A. and Gotz, M. (2002). Glial cells generate neurons: the role of the transcription factor Pax6. *Nat Neurosci* **5**, 308-15.

Hevner, R. F., Miyashita-Lin, E. and Rubenstein, J. L. (2002). Cortical and thalamic axon pathfinding defects in Tbr1, Gbx2, and Pax6 mutant mice: evidence that cortical and thalamic axons interact and guide each other. *J Comp Neurol* **447**, 8-17.

Hill, R. E., Favor, J., Hogan, B. L., Ton, C. C., Saunders, G. F., Hanson, I. M., Prosser, J., Jordan, T., Hastie, N. D. and van Heyningen, V. (1991). Mouse small eye results from mutations in a paired-like homeobox-containing gene. *Nature* **354**, 522-5.

Hogan, B. L., Hirst, E. M., Horsburgh, G. and Hetherington, C. M. (1988). Small eye (Sey): a mouse model for the genetic analysis of craniofacial abnormalities. *Development* **103 Suppl**, 115-9.

Holmes, G. P., Negus, K., Burridge, L., Raman, S., Algar, E., Yamada, T. and Little, M. H. (1998). Distinct but overlapping expression patterns of two vertebrate slit homologs implies functional roles in CNS development and organogenesis. *Mech Dev* **79**, 57-72.

Hong, K., Hinck, L., Nishiyama, M., Poo, M. M., Tessier-Lavigne, M. and Stein, E. (1999). A ligand-gated association between cytoplasmic domains of UNC5 and DCC family receptors converts netrin-induced growth cone attraction to repulsion. *Cell* **97**, 927-41.

Hopker, V. H., Shewan, D., Tessier-Lavigne, M., Poo, M. and Holt, C. (1999). Growth-cone attraction to netrin-1 is converted to repulsion by laminin-1. *Nature* **401**, 69-73.

Hussain, S. A., Piper, M., Fukuhara, N., Strochlic, L., Cho, G., Howitt, J. A., Ahmed, Y., Powell, A. K., Turnbull, J. E., Holt, C. E. et al. (2006). A molecular mechanism for the heparan sulfate dependence of slit-robo signaling. *J Biol Chem* **281**, 39693-8.

Islam, S. M., Shinmyo, Y., Okafuji, T., Su, Y., Naser, I. B., Ahmed, G., Zhang, S., Chen, S., Ohta, K., Kiyonari, H. et al. (2009). Draxin, a repulsive guidance protein for spinal cord and forebrain commissures. *Science* **323**, 388-93.

Jones, L., Lopez-Bendito, G., Gruss, P., Stoykova, A. and Molnar, Z. (2002). Pax6 is required for the normal development of the forebrain axonal connections. *Development* **129**, 5041-52.

Kantor, D. B., Chivatakarn, O., Peer, K. L., Oster, S. F., Inatani, M., Hansen, M. J., Flanagan, J. G., Yamaguchi, Y., Sretavan, D. W., Giger, R. J. et al. (2004). Semaphorin 5A is a bifunctional axon guidance cue regulated by heparan and chondroitin sulfate proteoglycans. *Neuron* **44**, 961-75.

Kastenhuber, E., Kern, U., Bonkowsky, J. L., Chien, C. B., Driever, W. and Schweitzer, J. (2009). Netrin-DCC, Robo-Slit, and heparan sulfate proteoglycans coordinate lateral positioning of longitudinal dopaminergic diencephalospinal axons. *J Neurosci* **29**, 8914-26.

Kawano, H., Fukuda, T., Kubo, K., Horie, M., Uyemura, K., Takeuchi, K., Osumi, N., Eto, K. and Kawamura, K. (1999). Pax-6 is required for thalamocortical pathway formation in fetal rats. *J Comp Neurol* **408**, 147-60.

Keino-Masu, K., Masu, M., Hinck, L., Leonardo, E. D., Chan, S. S., Culotti, J. G. and Tessier-Lavigne, M. (1996). Deleted in Colorectal Cancer (DCC) encodes a netrin receptor. *Cell* **87**, 175-85.

Keshishian, H. (1980). The origin and morphogenesis of pioneer neurons in the grasshopper metathoracic leg. *Dev Biol* **80**, 388-97.

Kessaris, N., Fogarty, M., Iannarelli, P., Grist, M., Wegner, M. and Richardson, W. D. (2006). Competing waves of oligodendrocytes in the forebrain and postnatal elimination of an embryonic lineage. *Nat Neurosci* **9**, 173-9.

Kidd, T., Bland, K. S. and Goodman, C. S. (1999). Slit is the midline repellent for the robo receptor in Drosophila. *Cell* **96**, 785-94.

Kiecker, C. and Lumsden, A. (2004). Hedgehog signaling from the ZLI regulates diencephalic regional identity. *Nat Neurosci* **7**, 1242-9.

Kitamura, K., Miura, H., Yanazawa, M., Miyashita, T. and Kato, K. (1997). Expression patterns of Brx1 (Rieg gene), Sonic hedgehog, Nkx2.2, Dlx1 and Arx during zona limitans intrathalamica and embryonic ventral lateral geniculate nuclear formation. *Mech Dev* **67**, 83-96.

Koester, S. E. and O'Leary, D. D. (1994). Axons of early generated neurons in cingulate cortex pioneer the corpus callosum. *J Neurosci* **14**, 6608-20.

Kolodkin, A. L., Matthes, D. J. and Goodman, C. S. (1993). The semaphorin genes encode a family of transmembrane and secreted growth cone guidance molecules. *Cell* **75**, 1389-99.

Kroll, T. T. and O'Leary, D. D. (2005). Ventralized dorsal telencephalic progenitors in Pax6 mutant mice generate GABA interneurons of a lateral ganglionic eminence fate. *Proc Natl Acad Sci U S A* **102**, 7374-9.

Leighton, P. A., Mitchell, K. J., Goodrich, L. V., Lu, X., Pinson, K., Scherz, P., Skarnes, W. C. and Tessier-Lavigne, M. (2001). Defining brain wiring patterns and mechanisms through gene trapping in mice. *Nature* **410**, 174-9.

Little, G. E., Lopez-Bendito, G., Runker, A. E., Garcia, N., Pinon, M. C., Chedotal, A., Molnar, Z. and Mitchell, K. J. (2009). Specificity and plasticity of thalamocortical connections in Sema6A mutant mice. *PLoS Biol* **7**, e98.

Lopez-Bendito, G., Cautinat, A., Sanchez, J. A., Bielle, F., Flames, N., Garratt, A. N., Talmage, D. A., Role, L. W., Charnay, P., Marin, O. et al. (2006). Tangential neuronal migration controls axon guidance: a role for neuregulin-1 in thalamocortical axon navigation. *Cell* **125**, 127-42.

Lopez-Bendito, G., Chan, C. H., Mallamaci, A., Parnavelas, J. and Molnar, Z. (2002). Role of Emx2 in the development of the reciprocal connectivity between cortex and thalamus. *J Comp Neurol* **451**, 153-69.

Lopez-Bendito, G., Flames, N., Ma, L., Fouquet, C., Di Meglio, T., Chedotal, A., Tessier-Lavigne, M. and Marin, O. (2007). Robo1 and Robo2 cooperate to control the guidance of major axonal tracts in the mammalian forebrain. *J Neurosci* **27**, 3395-407.

Lopez-Bendito, G. and Molnar, Z. (2003). Thalamocortical development: how are we going to get there? *Nat Rev Neurosci* **4**, 276-89.

Magnani, D., Hasenpusch-Theil, K., Jacobs, E. C., Campagnoni, A. T., Price, D. J. and Theil, T. (2010). The Gli3 hypomorphic mutation Pdn causes selective impairment in the growth, patterning, and axon guidance capability of the lateral ganglionic eminence. *J Neurosci* **30**, 13883-94.

Malatesta, P., Hartfuss, E. and Gotz, M. (2000). Isolation of radial glial cells by fluorescent-activated cell sorting reveals a neuronal lineage. *Development* **127**, 5253-63.

Manuel, M., Georgala, P. A., Carr, C. B., Chanas, S., Kleinjan, D. A., Martynoga, B., Mason, J. O., Molinek, M., Pinson, J., Pratt, T. et al. (2007). Controlled overexpression of Pax6 in vivo negatively autoregulates the Pax6 locus, causing cell-autonomous defects of late cortical progenitor proliferation with little effect on cortical arealization. *Development* **134**, 545-55.

Manuel, M. and Price, D. J. (2005). Role of Pax6 in forebrain regionalization. *Brain Res Bull* **66**, 387-93.

Marcos-Mondejar, P., Peregrin, S., Li, J. Y., Carlsson, L., Tole, S. and Lopez-Bendito, G. (2012). The lhx2 transcription factor controls thalamocortical axonal guidance by specific regulation of robo1 and robo2 receptors. *J Neurosci* **32**, 4372-85.

Marin, O. and Rubenstein, J. L. (2001). A long, remarkable journey: tangential migration in the telencephalon. *Nat Rev Neurosci* **2**, 780-90.

Mastick, G. S., Davis, N. M., Andrew, G. L. and Easter, S. S., Jr. (1997). Pax-6 functions in boundary formation and axon guidance in the embryonic mouse forebrain. *Development* **124**, 1985-97.

Mastick, G. S. and Easter, S. S., Jr. (1996). Initial organization of neurons and tracts in the embryonic mouse fore- and midbrain. *Dev Biol* **173**, 79-94.

Matsuo, T., Osumi-Yamashita, N., Noji, S., Ohuchi, H., Koyama, E., Myokai, F., Matsuo, N., Taniguchi, S., Doi, H., Iseki, S. et al. (1993). A mutation in the Pax-6 gene in rat small eye is associated with impaired migration of midbrain crest cells. *Nat Genet* **3**, 299-304.

McConnell, S. K., Ghosh, A. and Shatz, C. J. (1989). Subplate neurons pioneer the first axon pathway from the cerebral cortex. *Science* **245**, 978-82.

Medina, L., Legaz, I., Gonzalez, G., De Castro, F., Rubenstein, J. L. and Puelles, L. (2004). Expression of Dbx1, Neurogenin 2, Semaphorin 5A, Cadherin 8, and Emx1 distinguish ventral and lateral pallial histogenetic divisions in the developing mouse claustroamygdaloid complex. *J Comp Neurol* **474**, 504-23.

Metin, C. and Godement, P. (1996). The ganglionic eminence may be an intermediate target for corticofugal and thalamocortical axons. *J Neurosci* **16**, 3219-35.

Mire, E., Mezzera, C., Leyva-Diaz, E., Paternain, A. V., Squarzoni, P., Bluy, L., Castillo-Paterna, M., Lopez, M. J., Peregrin, S., Tessier-Lavigne, M. et al. (2012). Spontaneous activity regulates Robo1 transcription to mediate a switch in thalamocortical axon growth. *Nat Neurosci* **15**, 1134-43.

Mitrofanis, J. and Baker, G. E. (1993). Development of the thalamic reticular and perireticular nuclei in rats and their relationship to the course of growing corticofugal and corticopetal axons. *J Comp Neurol* **338**, 575-87.

Mitrofanis, J. and Guillery, R. W. (1993). New views of the thalamic reticular nucleus in the adult and the developing brain. *Trends Neurosci* **16**, 240-5.

Miyata, T., Kawaguchi, A., Saito, K., Kawano, M., Muto, T. and Ogawa, M. (2004). Asymmetric production of surface-dividing and non-surface-dividing cortical progenitor cells. *Development* **131**, 3133-45.

Molnar, Z., Adams, R. and Blakemore, C. (1998a). Mechanisms underlying the early establishment of thalamocortical connections in the rat. *J Neurosci* **18**, 5723-45.

Molnar, Z. and Blakemore, C. (1995). How do thalamic axons find their way to the cortex? *Trends Neurosci* **18**, 389-97.

Molnar, Z. and Cordery, P. (1999). Connections between cells of the internal capsule, thalamus, and cerebral cortex in embryonic rat. *J Comp Neurol* **413**, 1-25.

Molnar, Z., Knott, G. W., Blakemore, C. and Saunders, N. R. (1998b). Development of thalamocortical projections in the South American gray short-tailed opossum (Monodelphis domestica). *J Comp Neurol* **398**, 491-514.

Nacher, J., Varea, E., Blasco-Ibanez, J. M., Castillo-Gomez, E., Crespo, C., Martinez-Guijarro, F. J. and McEwen, B. S. (2005). Expression of the transcription factor Pax 6 in the adult rat dentate gyrus. *J Neurosci Res* **81**, 753-61.

Nakagawa, Y. and O'Leary, D. D. (2001). Combinatorial expression patterns of LIM-homeodomain and other regulatory genes parcellate developing thalamus. *J Neurosci* **21**, 2711-25.

Nakamoto, T., Kain, K. H. and Ginsberg, M. H. (2004). Neurobiology: New connections between integrins and axon guidance. *Curr Biol* **14**, R121-3.

Nguyen Ba-Charvet, K. T., Brose, K., Marillat, V., Kidd, T., Goodman, C. S., Tessier-Lavigne, M., Sotelo, C. and Chedotal, A. (1999). Slit2-Mediated chemorepulsion and collapse of developing forebrain axons. *Neuron* **22**, 463-73.

Noctor, S. C., Flint, A. C., Weissman, T. A., Dammerman, R. S. and Kriegstein, A. R. (2001). Neurons derived from radial glial cells establish radial units in neocortex. *Nature* **409**, 714-20.

Nural, H. F. and Mastick, G. S. (2004). Pax6 guides a relay of pioneer longitudinal axons in the embryonic mouse forebrain. *J Comp Neurol* **479**, 399-409.

Ottersen, O. P. (1980). Afferent connections to the amygdaloid complex of the rat and cat: II. Afferents from the hypothalamus and the basal telencephalon. *J Comp Neurol* **194**, 267-89.

Parras, C. M., Schuurmans, C., Scardigli, R., Kim, J., Anderson, D. J. and Guillemot, F. (2002). Divergent functions of the proneural genes Mash1 and Ngn2 in the specification of neuronal subtype identity. *Genes Dev* **16**, 324-38.

Pinon, M. C., Tuoc, T. C., Ashery-Padan, R., Molnar, Z. and Stoykova, A. (2008). Altered molecular regionalization and normal thalamocortical connections in cortex-specific Pax6 knock-out mice. *J Neurosci* **28**, 8724-34.

Piper, M., Plachez, C., Zalucki, O., Fothergill, T., Goudreau, G., Erzurumlu, R., Gu, C. and Richards, L. J. (2009). Neuropilin 1-Sema signaling regulates crossing of cingulate pioneering axons during development of the corpus callosum. *Cereb Cortex* **19 Suppl 1**, i11-21.

Placzek, M., Tessier-Lavigne, M., Jessell, T. and Dodd, J. (1990). Orientation of commissural axons in vitro in response to a floor plate-derived chemoattractant. *Development* **110**, 19-30.

Plump, A. S., Erskine, L., Sabatier, C., Brose, K., Epstein, C. J., Goodman, C. S., Mason, C. A. and Tessier-Lavigne, M. (2002). Slit1 and Slit2 cooperate to prevent premature midline crossing of retinal axons in the mouse visual system. *Neuron* **33**, 219-32.

Porteus, M. H., Bulfone, A., Liu, J. K., Puelles, L., Lo, L. C. and Rubenstein, J. L. (1994). DLX-2, MASH-1, and MAP-2 expression and bromodeoxyuridine incorporation define molecularly distinct cell populations in the embryonic mouse forebrain. *J Neurosci* **14**, 6370-83.

Powell, A. W., Sassa, T., Wu, Y., Tessier-Lavigne, M. and Polleux, F. (2008). Topography of thalamic projections requires attractive and repulsive functions of Netrin-1 in the ventral telencephalon. *PLoS Biol* **6**, e116.

Pratt, T., Quinn, J. C., Simpson, T. I., West, J. D., Mason, J. O. and Price, D. J. (2002). Disruption of early events in thalamocortical tract formation in mice lacking the transcription factors Pax6 or Foxg1. *J Neurosci* **22**, 8523-31.

Pratt, T., Sharp, L., Nichols, J., Price, D. J. and Mason, J. O. (2000a). Embryonic stem cells and transgenic mice ubiquitously expressing a tau-tagged green fluorescent protein. *Dev Biol* **228**, 19-28.

Pratt, T., Vitalis, T., Warren, N., Edgar, J. M., Mason, J. O. and Price, D. J. (2000b). A role for Pax6 in the normal development of dorsal thalamus and its cortical connections. *Development* **127**, 5167-78.

Price, D. J., Clegg, J., Duocastella, X. O., Willshaw, D. and Pratt, T. (2012). The importance of combinatorial gene expression in early Mammalian thalamic patterning and thalamocortical axonal guidance. *Front Neurosci* **6**, 37.

Price, D. J., Kennedy, H., Dehay, C., Zhou, L., Mercier, M., Jossin, Y., Goffinet, A. M., Tissir, F., Blakey, D. and Molnar, Z. (2006). The development of cortical connections. *Eur J Neurosci* **23**, 910-20.

Puelles, L. and Rubenstein, J. L. (2003). Forebrain gene expression domains and the evolving prosomeric model. *Trends Neurosci* **26**, 469-76.

Quinn, J. C., Molinek, M., Martynoga, B. S., Zaki, P. A., Faedo, A., Bulfone, A., Hevner, R. F., West, J. D. and Price, D. J. (2007). Pax6 controls cerebral cortical cell number by regulating exit from the cell cycle and specifies cortical cell identity by a cell autonomous mechanism. *Dev Biol* **302**, 50-65.

Quinn, J. C., West, J. D. and Hill, R. E. (1996). Multiple functions for Pax6 in mouse eye and nasal development. *Genes Dev* **10**, 435-46.

Quiring, R., Walldorf, U., Kloter, U. and Gehring, W. J. (1994). Homology of the eyeless gene of Drosophila to the Small eye gene in mice and Aniridia in humans. *Science* **265**, 785-9.

Rothberg, J. M., Hartley, D. A., Walther, Z. and Artavanis-Tsakonas, S. (1988). slit: an EGF-homologous locus of D. melanogaster involved in the development of the embryonic central nervous system. *Cell* **55**, 1047-59.

Rothberg, J. M., Jacobs, J. R., Goodman, C. S. and Artavanis-Tsakonas, S. (1990). slit: an extracellular protein necessary for development of midline glia and commissural axon pathways contains both EGF and LRR domains. *Genes Dev* **4**, 2169-87.

Sabry, J. H., O'Connor, T. P., Evans, L., Toroian-Raymond, A., Kirschner, M. and Bentley, D. (1991). Microtubule behavior during guidance of pioneer neuron growth cones in situ. *J Cell Biol* **115**, 381-95.

Sanchez-Camacho, C. and Bovolenta, P. (2009). Emerging mechanisms in morphogen-mediated axon guidance. *Bioessays* **31**, 1013-25.

Sansom, S. N., Griffiths, D. S., Faedo, A., Kleinjan, D. J., Ruan, Y., Smith, J., van Heyningen, V., Rubenstein, J. L. and Livesey, F. J. (2009). The level of the transcription factor Pax6 is essential for controlling the balance between neural stem cell self-renewal and neurogenesis. *PLoS Genet* **5**, e1000511.

Schmahl, W., Knoedlseder, M., Favor, J. and Davidson, D. (1993). Defects of neuronal migration and the pathogenesis of cortical malformations are associated with Small eye (Sey) in the mouse, a point mutation at the Pax-6-locus. *Acta Neuropathol* **86**, 126-35.

Seeger, M., Tear, G., Ferres-Marco, D. and Goodman, C. S. (1993). Mutations affecting growth cone guidance in Drosophila: genes necessary for guidance toward or away from the midline. *Neuron* **10**, 409-26.

Serafini, T., Colamarino, S. A., Leonardo, E. D., Wang, H., Beddington, R., Skarnes, W. C. and Tessier-Lavigne, M. (1996). Netrin-1 is required for commissural axon guidance in the developing vertebrate nervous system. *Cell* **87**, 1001-14.

Serafini, T., Kennedy, T. E., Galko, M. J., Mirzayan, C., Jessell, T. M. and Tessier-Lavigne, M. (1994). The netrins define a family of axon outgrowth-promoting proteins homologous to C. elegans UNC-6. *Cell* **78**, 409-24.

Shirasaki, R., Katsumata, R. and Murakami, F. (1998). Change in chemoattractant responsiveness of developing axons at an intermediate target. *Science* **279**, 105-7.

Shu, T., Sundaresan, V., McCarthy, M. M. and Richards, L. J. (2003). Slit2 guides both precrossing and postcrossing callosal axons at the midline in vivo. *J Neurosci* **23**, 8176-84.

Shu, T., Valentino, K. M., Seaman, C., Cooper, H. M. and Richards, L. J. (2000). Expression of the netrin-1 receptor, deleted in colorectal cancer (DCC), is largely confined to projecting neurons in the developing forebrain. *J Comp Neurol* **416**, 201-12.

Simpson, T. I., Pratt, T., Mason, J. O. and Price, D. J. (2009). Normal ventral telencephalic expression of Pax6 is required for normal development of thalamocortical axons in embryonic mice. *Neural Dev* **4**, 19.

Skaliora, I., Singer, W., Betz, H. and Puschel, A. W. (1998). Differential patterns of semaphorin expression in the developing rat brain. *Eur J Neurosci* **10**, 1215-29.

Song, H. J., Ming, G. L. and Poo, M. M. (1997). cAMP-induced switching in turning direction of nerve growth cones. *Nature* **388**, 275-9.

Sousa, V. H., Miyoshi, G., Hjerling-Leffler, J., Karayannis, T. and Fishell, G. (2009). Characterization of Nkx6-2-derived neocortical interneuron lineages. *Cereb Cortex* **19 Suppl 1**, i1-10.

St-Onge, L., Sosa-Pineda, B., Chowdhury, K., Mansouri, A. and Gruss, P. (1997). Pax6 is required for differentiation of glucagon-producing alpha-cells in mouse pancreas. *Nature* **387**, 406-9.

Stein, E. and Tessier-Lavigne, M. (2001). Hierarchical organization of guidance receptors: silencing of netrin attraction by slit through a Robo/DCC receptor complex. *Science* **291**, 1928-38.

Stoykova, A., Fritsch, R., Walther, C. and Gruss, P. (1996). Forebrain patterning defects in Small eye mutant mice. *Development* **122**, 3453-65.

Stoykova, A., Gotz, M., Gruss, P. and Price, J. (1997). Pax6-dependent regulation of adhesive patterning, R-cadherin expression and boundary formation in developing forebrain. *Development* **124**, 3765-77.

Stoykova, A. and Gruss, P. (1994). Roles of Pax-genes in developing and adult brain as suggested by expression patterns. *J Neurosci* **14**, 1395-412.

Stoykova, A., Treichel, D., Hallonet, M. and Gruss, P. (2000). Pax6 modulates the dorsoventral patterning of the mammalian telencephalon. *J Neurosci* **20**, 8042-50.

Suto, F., Ito, K., Uemura, M., Shimizu, M., Shinkawa, Y., Sanbo, M., Shinoda, T., Tsuboi, M., Takashima, S., Yagi, T. et al. (2005). Plexin-a4 mediates axon-repulsive activities of both secreted and transmembrane semaphorins and plays roles in nerve fiber guidance. *J Neurosci* **25**, 3628-37.

Talamillo, A., Quinn, J. C., Collinson, J. M., Caric, D., Price, D. J., West, J. D. and Hill, R. E. (2003). Pax6 regulates regional development and neuronal migration in the cerebral cortex. *Dev Biol* **255**, 151-63.

Tamada, A., Kumada, T., Zhu, Y., Matsumoto, T., Hatanaka, Y., Muguruma, K., Chen, Z., Tanabe, Y., Torigoe, M., Yamauchi, K. et al. (2008). Crucial roles of Robo proteins in midline crossing of cerebellofugal axons and lack of their up-regulation after midline crossing. *Neural Dev* **3**, 29.

Taniguchi, M., Yuasa, S., Fujisawa, H., Naruse, I., Saga, S., Mishina, M. and Yagi, T. (1997). Disruption of semaphorin III/D gene causes severe abnormality in peripheral nerve projection. *Neuron* **19**, 519-30.

Tarabykin, V., Stoykova, A., Usman, N. and Gruss, P. (2001). Cortical upper layer neurons derive from the subventricular zone as indicated by Svet1 gene expression. *Development* **128**, 1983-93.

Tessier-Lavigne, M., Placzek, M., Lumsden, A. G., Dodd, J. and Jessell, T. M. (1988). Chemotropic guidance of developing axons in the mammalian central nervous system. *Nature* **336**, 775-8.

Tian, N. M., Pratt, T. and Price, D. J. (2008). Foxg1 regulates retinal axon pathfinding by repressing an ipsilateral program in nasal retina and by causing optic chiasm cells to exert a net axonal growth-promoting activity. *Development* **135**, 4081-9.

Tissir, F., Bar, I., Jossin, Y., De Backer, O. and Goffinet, A. M. (2005). Protocadherin Celsr3 is crucial in axonal tract development. *Nat Neurosci* **8**, 451-7.

Ton, C. C., Hirvonen, H., Miwa, H., Weil, M. M., Monaghan, P., Jordan, T., van Heyningen, V., Hastie, N. D., Meijers-Heijboer, H., Drechsler, M. et al. (1991).

Positional cloning and characterization of a paired box- and homeobox-containing gene from the aniridia region. *Cell* **67**, 1059-74.

Toresson, H., Potter, S. S. and Campbell, K. (2000). Genetic control of dorsal-ventral identity in the telencephalon: opposing roles for Pax6 and Gsh2. *Development* **127**, 4361-71.

Tsuchiya, R., Takahashi, K., Liu, F. C. and Takahashi, H. (2009). Aberrant axonal projections from mammillary bodies in Pax6 mutant mice: possible roles of Netrin-1 and Slit 2 in mammillary projections. *J Neurosci Res* **87**, 1620-33.

Tuoc, T. C., Radyushkin, K., Tonchev, A. B., Pinon, M. C., Ashery-Padan, R., Molnar, Z., Davidoff, M. S. and Stoykova, A. (2009). Selective cortical layering abnormalities and behavioral deficits in cortex-specific Pax6 knock-out mice. *J Neurosci* **29**, 8335-49.

Tuttle, R., Nakagawa, Y., Johnson, J. E. and O'Leary, D. D. (1999). Defects in thalamocortical axon pathfinding correlate with altered cell domains in Mash-1-deficient mice. *Development* **126**, 1903-16.

Tyas, D. A., Simpson, T. I., Carr, C. B., Kleinjan, D. A., van Heyningen, V., Mason, J. O. and Price, D. J. (2006). Functional conservation of Pax6 regulatory elements in humans and mice demonstrated with a novel transgenic reporter mouse. *BMC Dev Biol* **6**, 21.

Vue, T. Y., Aaker, J., Taniguchi, A., Kazemzadeh, C., Skidmore, J. M., Martin, D. M., Martin, J. F., Treier, M. and Nakagawa, Y. (2007). Characterization of progenitor domains in the developing mouse thalamus. *J Comp Neurol* **505**, 73-91.

Vue, T. Y., Bluske, K., Alishahi, A., Yang, L. L., Koyano-Nakagawa, N., Novitch, B. and Nakagawa, Y. (2009). Sonic hedgehog signaling controls thalamic progenitor identity and nuclei specification in mice. *J Neurosci* **29**, 4484-97.

Walther, C. and Gruss, P. (1991). Pax-6, a murine paired box gene, is expressed in the developing CNS. *Development* **113**, 1435-49.

Wang, H. F. and Liu, F. C. (2001). Developmental restriction of the LIM homeodomain transcription factor Islet-1 expression to cholinergic neurons in the rat striatum. *Neuroscience* **103**, 999-1016.

Warren, N., Caric, D., Pratt, T., Clausen, J. A., Asavaritikrai, P., Mason, J. O., Hill, R. E. and Price, D. J. (1999). The transcription factor, Pax6, is required for cell proliferation and differentiation in the developing cerebral cortex. *Cereb Cortex* **9**, 627-35.

Warren, N. and Price, D. J. (1997). Roles of Pax-6 in murine diencephalic development. *Development* **124**, 1573-82.

Weiss, P. A. (1934). In vitro experiments on the factors determining the course of the outgrowing nerve
fiber. *J. Exp. Zool* **68**, 393-448.

Wichterle, H., Alvarez-Dolado, M., Erskine, L. and Alvarez-Buylla, A. (2003). Permissive corridor and diffusible gradients direct medial ganglionic eminence cell migration to the neocortex. *Proc Natl Acad Sci U S A* **100**, 727-32.

Winberg, M. L., Noordermeer, J. N., Tamagnone, L., Comoglio, P. M., Spriggs, M. K., Tessier-Lavigne, M. and Goodman, C. S. (1998). Plexin A is a neuronal semaphorin receptor that controls axon guidance. *Cell* **95**, 903-16.

Wright, A. G., Demyanenko, G. P., Powell, A., Schachner, M., Enriquez-Barreto, L., Tran, T. S., Polleux, F. and Maness, P. F. (2007). Close homolog of L1 and neuropilin 1 mediate guidance of thalamocortical axons at the ventral telencephalon. *J Neurosci* **27**, 13667-79.

Yun, K., Potter, S. and Rubenstein, J. L. (2001). Gsh2 and Pax6 play complementary roles in dorsoventral patterning of the mammalian telencephalon. *Development* **128**, 193-205.